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Dynamical Darwinian selection of a more productive strain of *Tisochrysis lutea*

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ABSTRACT

Species domestication and improvement were decisive in increasing agricultural production yields. The microalgae industry must now realize the same accomplishment to boost its development instead of only using wild strains. Although genetically engineering microalgae is a promising path to explore, the artificial modification of the microalgae genome can have adverse side-effects on biomass productivity. Here, we propose a Darwinian method to select and improve microalgal strains, exploiting the competitive exclusion principle in a dynamical environment to drive evolution. Choosing an appropriate selection pressure allows a new adapted population having enhanced properties to emerge. In a natural growing environment, light intensity is consistently changing due to meteorological events, differing microalgae concentration and other exogenous factors. Consequently, the pigmentary profile regulation and the associated genetic mechanism is highly modulable in most microalgae. On top of this, high light intensities are known to elicit strong cellular stresses. For these reasons, a dynamical light regime with high light intensities was deemed a potentially efficient selection factor for the emergence of a new strain adapted to higher irradiance levels. Following 160 days of selection under variable light intensity the biomass productivity of *Tisochrysis lutea* increased by 77%. Genetic analyses further confirmed the selection protocol's success, with the apparition of 2716 new alleles. To our knowledge, this is the first selection approach allowing a significant biomass productivity increase resulting from an enhanced capacity of microalgae pigment photoadaptation.

1. Introduction

Microalgae offer promising green industrial applications in animal feed, foods, cosmetics with further prospects for green chemistry and biofuels [1]. Until now, the exploited strains were wild types with poor industrial and economic performances [2]. To mimic the agricultural field, more efficient strains with improved characteristics must be selected to enhance biomass productivity, and hence, profitability of microalgal biorefineries.

To be economically attractive at an industrial scale, microalgal cultures must be conducted at high biomass density [3,4], but dense cultures lead to a highly heterogeneous light distribution. Light intensity (or irradiance) in dense cultures strongly decreases toward the core of the bioreactors, where less than 15% of the incident light is still available [5]. Besides, cells are photoinhibited when close to the light source, whereas they are photolimited in most of the reactor, overall leading to a reduced biomass productivity [6].

Light absorption in microalgal cultures is mainly due to pigments that photoacclimation processes dynamically regulate [7,8]. A shift from high to low light intensity induces an increase in primary pigments quantity such as chlorophyll *a* [7,9]. Accessory pigments such as chlorophyll *c* in the prymnesiophyte *Tisochrysis lutea* evolve similarly [8,10]. These pigment changes result from substantial modifications in the photosynthetic apparatus [11]. Depending on the species, the total amount of photosystems I (PS I) and II (PS II) or their sizes increase in response to light limitation. Microalgae become less rich in pigments and more sensitive to photoinhibition [8] as the absorption cross-section increases [12–15]. Similarly, for secondary pigments such as

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xanthophylls, a shift from high to low irradiance generally decreases their intracellular content, as observed in *Tisochrysis lutea* [8,10]. This behavior can be species-dependent, an increase was for instance reported in *Nitzschia palea* [16]. The photoacclimation kinetics are also species-dependent [7], with a time scale from one to several days [14].

The selection of cells with smaller photosynthetic antenna (*i.e.* less photosynthetic pigments) appears to be a promising way to reduce photoinhibition and better distribute light in the reactor, leading to a higher biomass and enhanced productivity [17,18]. Different approaches were carried out to reduce photosynthetic antenna sizes (Table 1). For instance, cells were selected and isolated based on their fluorescence and pigmentary properties after a random mutagenesis step (with UV or chemicals) to create genetic diversity ([19–21], Table 1). Direct genome modification (GMO) to reduce the antenna size was also tested [22]. By modifying the *tla1* gene with plasmid insertion, Polle et al. obtained a chlorophyll deficient mutant. These GMO approaches successfully led to lower chlorophyll content and reduced photosynthetic antenna size, resulting in higher biomass for identical irradiance. For example, Polle et al. [22] observed a sharp decline of 166% in chlorophyll content and a sixfold increase in biomass content in the *tla1* mutant strain compared to the wild type (Table 1). Unfortunately, such GMO also appeared deficient in chlorophyll *a* and chlorophyll *b* binding proteins (essential for photoprotection and energy conversion), resulting in a barely enhanced biomass productivity [17]. Random mutagenesis also leads to the modification of various genes and to deleterious side effects on growth [17,21].

Here, we propose an alternative mode of selection based on the competitive exclusion principle [28]. This principle postulates that individuals with a higher growth rate stay in the reactor within a continuous culture submitted to given selection stress. In contrast, the other individuals, less tolerant to this condition are progressively eliminated [29]. This method has already given probative results for organisms such as bacteria (Bennett et al.) and yeast (Brown and Oliver; Jiménez and Benítez) but was barely considered for microalgae.

In this study, we hypothesized that a prolonged exposure of microalgae to a variable light regime can lead to the selection of individuals with a modified pigment composition. The principle of this mode of selection is based on the observation that the more chlorophyll *a* a cell contains, the more prone to photoinhibition it is [8]. Consequently, successive episodes of high light intensity should have more harmful effects on the cells richer in chlorophyll *a*nd their growth. In an open culture not limited by nutrients (turbidostat), this differential effect will progressively lead to the domination of cells altering their pigmentary profile and concentration to an optimum level, and finally to selecting individuals better coping with changing light conditions.

The difficulty of such long-term experiments essentially lies in the choice of an appropriate operating mode. In the present case, cells must survive in a light regime alternating photoinhibition and resting phases, with permanent photoacclimation. To optimize the selection process, two conditions were implemented:

- First, according to Combe et al. [30], cells subjected to a variable light regime adjust their photosynthetic apparatus to the average light level. Thus, in our case, maintaining the average light intensity at a low level throughout the experiment promoted the synthesis of chlorophyll due to photoacclimation, and the photoinhibition induced by the light episodes was more nocuous.
- The second condition consisted of gradually increasing the stress intensity. The excess of harvested energy in the cell can lead to the destruction of key proteins like the D1 protein involved in the photosystem II. More generally, the flux of electrons and cofactors can generate an oxidative stress in the cell inducing different damages. This is also combined with the higher oxygen production, leading to the increase of free radicals [31]. Photoinhibition episodes were then progressively intensified to increase selection pressure on the surviving individuals. This kind of "ratchet" protocol proved effective to enhance the acclimation of microorganisms to different stresses [32].

The microalgae species chosen in this study was *Tisochrysis lutea* (formerly *Isochrysis galbana affinis* Tahiti (*T-iso*)). This oleaginous *Prymnesiophyceae* is commonly used in aquaculture [33] as a source of docosahexaenoic acid (DHA) [34–36]. The strain used in this work (named W2X) resulted from previous mutation/selection processes of the wild-type strain (see [2,37]) and produces twice as many neutral lipids (mainly triglycerides) when compared to the wild type. W2X being a non-monoclonal strain, the corresponding microalgae population possesses a pre-existing genotypic diversity, likely enhancing the selection efficiency [38]. *Tisochrysis lutea* has already been used for varietal selection experiments, where temperature was chosen as the selection pressure, leading to an increase of the thermal niche, neutral lipid production [39], and PUFA composition of polar lipids [31]. To conduct this type of experiment, it is necessary to have a highly reliable device that can control and monitor continuous cultures in real time for several months without interruption. In this study, this device, called a selectiostat, a plane photobioreactors of 1.9 L designed to grow microalgae under long-term selection pressure, was adapted so that the light intensity was the stress factor.

Here, we designed a novel light-driven selection procedure to obtain a strain adapted to drastic high light intensity changes. After 160 days of selection, a comparison of the initial *T-iso* strain (W2X) with the selected one (S2X) in similar culturing conditions revealed an 77% biomass productivity increase (measured as the total carbon productivity). Analysis of the initial W2X and final selected S2X pigmentary profile showed a reorganization of the photosynthetic and photoprotective pigments, possibly explaining the higher biomass productivity. Finally, a comparison of W2X and S2X genome was carried out and confirmed the obtention of a modified organism by using a dynamical Darwinian selection protocol.

Table 1
Review of various attempts for reducing microalgae photosynthetic antenna size. The ratio of new strain/wild type is used to compare with normalized data.

Targeted Gene	Microalgae	Increase in chlorophyll *a* content between WT and new strain	Biomass productivity increase	Reference
–	*Tisochrysis lutea*	0.65	1.79	*This study*
TAM2	*Chlorella sorokiniana*	0.63	1.32	[23]
E2	*Nannochloropsis gaditana*	0.50	1.25	[21]
LCH-1	*Chlamydomonas perigranulata*	0.42	1.22	[24]
Stm3LR3	*Chlamydomonas reinhardtii*	0.32	1.00	([17]; [25])
TLA2	*Chlamydomonas reinhardtii*	0.17	0.95	([17]; [26])
CM1–1	*Cyclotella sp.*	0.43	0.85	[19]
AS2.2	*Chlamydomonas reinhardtii*	1.00	0.77	([27]; [17])
BF4	*Chlamydomonas reinhardtii*	No data	0.75	[17]
Tla1	*Chlamydomonas reinhardtii*	0.37	No data	[22]
Tla3	*Chlamydomonas reinhardtii*	0.14	No data	[12]

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2. Materials and methods

2.1. Microalgae strain

The domesticated microalgae *Tisochrysis lutea* (CCAP 927/17) was chosen.

2.2. Culturing system for the selection process (selectiostat)

Two continuous biological replicate cultures were run in turbidostat mode ($n = 2$). The enrichment medium (f/2 medium, [40]) was prepared in 20 L tanks (Nalgen) filled with three weeks-matured natural seawater, previously filtered on 0.1 μm, and autoclaved at 120 °C for 40 min. After cooling, the medium was added through a sterile filter. Cultures were continuously and gently homogenized by a magnetic stirrer bar and air bubbling. The maximum biomass density attainable in the photobioreactor was estimated at 3.3×10^6 cells.mL^{-1}. The biomass concentration threshold was set at 8.7×10^5 cells.mL^{-1}. The dilution rate was controlled by the ODIN® software [41] according to the turbidity measured on-line at 800 nm.

2.3. Cleaning procedure

Biofilm was removed monthly (over the 160 days of selection), according to the following procedure. 1 L of selection culture was taken and kept in an autoclaved Schott bottle. After that, selectiostats were disassembled and washed with Milli-Q water and 70% ethanol. Once dried and reassembled, the photobioreactors were sterilized with 10% HCl and rinsed with fresh sterile medium filtered through 0.22 μm (SpiralCap, Gelman). Selectiostats were then inoculated with the preserved culture and complemented with sterile medium filtered through 0.22 μm with a Stepdos pump (KNF) (up to) to a volume of 1.9 L.

2.4. Automatic control procedure

pH was regulated at 8.2 by computer-controlled micro-additions of CO_2. Temperature was maintained using a double water jacket connected to a cryostat (Lauda Proline RP845) set at 28.2 °C. Cultures were illuminated with LEDs (Nichia NVSL219BT 2700 K) placed on one side of the photobioreactors. Light intensity was continuously measured with a probe (SKY, SKL2620) placed on the LEDs' opposing side of the reactor. Light, pH, and temperature, were continuously monitored by ODIN® software [41].

2.5. Continuous selection experiment, the so-called ratchet protocol

The selection was conducted based on a modified protocol from [39]. Periodic shifts between low and high irradiance levels (see Table 2) were employed as a selection pressure. Dark and bright cycles lasted between 1.5 and 2 h according to the light intensity of the bright phase (Table 2). Durations of *low-* and *high-light* phases (t_{LL}, t_{HL}), as well as irradiance levels (I_{HL}), were chosen to maintain a low average light intensity (I_{av}, defined as $I_{av} = \frac{I_{HL}*t_{HL}+I_{LL}*t_{LL}}{t_{HL}+t_{LL}}$). t_{LL}, t_{HL} were increased or decreased to keep I_{av} to the desired value at a specified I_{HL}. Light patterns were kept unchanged for 14 days. This duration, later called the *selection cycle*, was sufficient for cultures to reach a steady growth rate. At the end of a selection cycle, if the average growth rate was higher than 0.4 d^{-1} (50% of the maximal growth rate observed for the initial strain in the selectiostat (conditions described in the section "Benchmarking experiments"), a new selection cycle was then initiated and new values for I_{HL}, t_{HL}, and t_{LL} were set. I_{HL} was chosen to strengthen the selection pressure, and t_{HL} and t_{LL} values were adjusted to keep I_{av} as low as possible. The difference ($I_{HL} - I_{av}$) that characterizes the selection pressure was gradually increased over the five consecutive selection cycles (Table 2).

Table 2
Light conditions imposed on microalgae during the selection experiment.

Cycle	I_{HL} (μmol.m^{-2}.s^{-1})	I_{av} (μmol.m^{-2}.s^{-1})	I_{HL}- I_{av} (μmol.m^{-2}.s^{-1})	HL exposure time t_{HL} (s)	Dark exposure time t_{LL} (s)
1	800	267	600	1800	3600
2	1000	300	700	2160	5040
3	1200	350	850	2100	5100
4	1400	350	1050	1800	5400
5	1600	400	1200	1800	5400

The number of generations (N) during the whole experiment was calculated as follows:

$$N = \frac{\mu_{average}\, T}{\ln(2)} \quad (1)$$

where $\mu_{average}$ is the average growth rate calculated over the duration T of the whole experiment.

2.6. Benchmark experiments to monitor the evolution in the strains

A benchmark batch-mode culturing device working in standardized conditions was set-up to monitor the population evolution weekly. Two 2 L double-jacketed cylindrical glass photobioreactors were used as biological replicate ($n = 2$). The temperature was controlled at 28.2 °C, pH maintained constant at 8.3 by micro-additions of pure CO_2, and continuous light (200 μmol.m^{-2}.s^{-1}, measured at the center of the empty photobioreactors) was provided by fluorescent tubes (Dulux®1, 2G11, 55 W/12–950, lumilux de lux, daylight, OsramSylvania). Cultures were homogenized by gentle magnetic stirring and filtrated air bubbling. Each week, 200 mL of the selectiostat cultures were transferred into the benchmark photobioreactors. The same volume of f/2-enriched [40] fresh sterile medium was added to prevent nutrient limitation. This dilution also prevented light limitation (transmittance >18% after four days). Four days after inoculation, pigments were measured as described in [42].

Cell optical cross-section for absorption σ_a at 680 nm (m^2.cell^{-1}) was measured in triplicate to obtain an estimation of the photosynthesis apparatus size. *In vivo* measurements of bulk spectral absorption, a(λ), were performed between 400 and 800 nm on culture samples of 1×10^6 cells.mL^{-1} with a spectrophotometer (Varian, DMS100) equipped with an integrating sphere. Optical cross-section for absorption σ_a (680 nm) was obtained by normalizing the bulk absorption a (680 nm) to the measured cell concentration [43].

2.7. Final comparison between the initial and adapted strains (conclusive experiment)

At the end of the selection experiment, batch cultures were carried out to compare the characteristics of the initial W2× and adapted S2× strains. W2× was grown in biological duplicate, and S2× duplicate strain was grown in biological quadruplicate (2 × 2). The culture conditions were close to the selection experiment (pH regulated at 8.2; temperature at 28 °C). Cultures were illuminated on one side by arrays of 50 cm fluorescent tubes (Dulux®1, 2G11, 55 W/12–950, lumilux de lux, daylight, OsramSylvania), delivering a light intensity I_{out} of 130 ± 12 μmol.m^{-2}.s^{-1} measured in the empty vessels. After five days of acclimation to these light conditions, cultures were diluted with fresh medium to $5.1 \pm 0.8 \times 10^4$ cells.mL^{-1}. The batch cultures were monitored for 13 days. To ensure that nutrients were not limiting (later verified by C/N measurements), a new concentrated sterile enrichment medium was regularly added. Cell and carbon concentrations were measured in triplicate measurements ($n = 3$) twice a day [42]. The maximum division rate μ_{max} was measured during the exponential

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growth phase (later referred to as phase I) according to the following equation:

$$\mu_{max} = \frac{Ln\left(\frac{Cell_2}{Cell_1}\right)}{t_2 - t_1} \quad (2)$$

where $Cell_1$ and $Cell_2$ are the cell concentrations (cell.ml^{-1}) at time t_1 and t_2, respectively. During the rest of the culturing time (later called phase II), the growth rate progressively decreased, as generally observed in batch cultures.

The total carbon productivity (P) (μgC.mL^{-1}.d^{-1}) of the S2X and W2X strains was calculated as follows:

$$P = \frac{\frac{C_f}{C_i}}{t} \quad (3)$$

where C_i and C_f are the microalgae carbon concentrations (μgC.ml^{-1}) measured at the beginning and the end of the batch cultures, respectively, and t the time between the final and initial measurements in days (d^{-1}).

Sampling was made at the beginning of phase 1 and the end of phase 2 (Fig. 2) for pigment measurements. 5.75 ml of culture were filtered in triplicates onto pre-combusted glass-fiber filters (450 °C, 12 h, Whatman GF/C) and kept at −80 °C until analysis. HPTLC analyses was performed on the filtered microalgae residue in triplicate measurements (n = 3), as previously described [42].

2.8. DNA extraction, sequencing, mapping, and single nucleotide polymorphisms (SNPs) detections method

The DNA was extracted using a phenol-chloroform method, described in detail in [44] for W2X and S2X strains. For each strain, 4 μg of DNA were used for a whole sequencing with an Illumina HiSeq3000 (2×150b) at the GenoToul Platform. The raw sequencing data obtained was: i) filtered to eliminate Illumina residual adapters with cutadapt software [45] ii) only read in pairs with a mean sequencing quality score higher than Q30 were conserved and iii) reads with length higher than 100 bases for both reads of pair were conserved. After these filtering steps, 21,334,865 and 12,520,340 of reads pairs from W2X and S2X strains were respectively obtained. Reads pairs were mapped into the Tiso reference genomeV2 [46] using BWA software [47]. On average, 92.5% of the reads were aligned on the reference genome for a depth mean 68.2X and 42.2X, respectively, for W2X and S2X strains. Identification of SNPs was performed using Freebayes software [48] (minimal total coverage: 20; minimal allele coverage: 6; pooled-continuous option as the number of individuals in strains was unknown). SNiPlay software [49] was used to compare strains to identify mutual or specific alleles.

2.9. Statistics

Standard variation was measured on 6 replicates minimum (including biological replicates $n = 2$ minimum and measurement replicates $n = 3$ minimum). Regressions were made using sigma plot. The hypothesis of linear correlation was accepted considering a p-value <0.05.

3. Results and discussion

3.1. Light-driven Darwinian selection of the initial strain (W2X), microalgal population monitoring during the selection procedure

The selection experiment lasted 160 days, corresponding to about 120 generations. Intracellular chlorophyll a concentration, measured during the weekly benchmark experiments, progressively decreased at a mean rate of $1.5 \pm 0.24 \times 10^{-3}$ pgChl a.cell^{-1}.d^{-1} (Fig. 1; $n = 20$; p-value <0.01). Simultaneously, the optical cross-section for absorption, inversely correlated to antenna size, decreased by $4.2 \pm 0.74 \times 10^{-14}$ m^2.cell^{-1}.d^{-1} (n = 20; p-value <0.01). The normalization of these values by their averages (*i.e.*, 2.35×10^{-12} m^2.cell^{-1} and 1.49×10^{-1} pgChl a.cell^{-1} for optical cross-section and chlorophyll a content, respectively) provides an estimate of the adaptation rate (1.01 and 1.79 d^{-1} for cell chlorophyll a and optical cross-section, respectively). The optical cross-section for absorption was modified faster than the chlorophyll a content, leading to the observed curvilinear evolution in Fig. 1. The curvilinear aspect of the relationship between the optical cross-section and chlorophyll a reflects the packaging effect due to its spatial reorganization in the cells [50]. At the end of the selection experiment, the resulting S2X strain exhibited a new phenotype with less chlorophyll a under the *Benchmark* conditions and thus absorbing less light. To further confirm the success of the protocol, a genomic comparison between the selected strain S2X and the initial strain W2X was performed.

3.2. Genomic comparison of the initial strain, W2X and the selected strain, S2X

The genomes of the W2X and S2X strains were sequenced to analyze the genetic impact of the selection procedure and support the idea that a new strain emerged. First, polyallelic *loci* were identified in the genome of each strain to estimate the genetic diversity. The number of polyallelic *loci* was 13,243 single nucleotide polymorphisms (SNPs) and 12,694 SNPs for W2X and S2X strains, respectively. Furthermore, the selection parameters herein used did not lead to the emergence of a pure lineage strain. The allelic diversity was conserved in the S2X strain despite the applied selection procedure.

Identified polymorphisms (SNPs) were compared to understand genomic evolution, which led to the emergence of a new strain (Table 3). Between both W2X and S2X, 7325 poly-allelic loci were conserved despite the selection procedure. As expected, neutral or unfavorable alleles (4064) were lost while new alleles (2772) appeared during the selection procedure. Among new alleles, 56 were fixed entirely in the S2X strain (present in 100% individuals from the population), which likely played a role in this adaptation. However, none of the candidate genes with polymorphism (385 SNPs were located in coding regions distributed within 254 genes) can clearly explain the observed phenotypic differences in the S2X strain (Supp data). Nevertheless, functional annota-

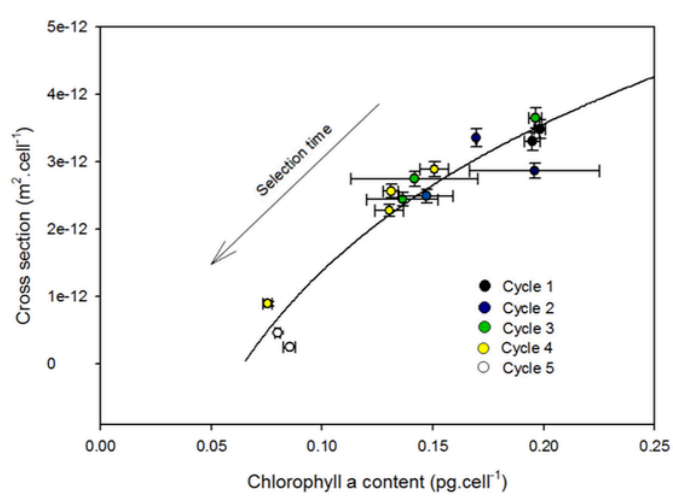

Fig. 1. Relationship between optical cross-section for absorption and cell chlorophyll a measured within the independent benchmark experiments at the end of each selection cycle. The arrow indicates selection time. Cross section and chlorophyll content were performed in triplicate on the two biological duplicates (n = 2).

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Table 3
Genetic impact of the selection procedure and changes between W2X and S2X for one locus.

Entry	Genotype evolution for one locus		Origin of polymorphism in S2X	Number of polymorphisms
	W2X	S2X		
1	(1:1)	(0:1)	Selection effect, partially fix allele	197
2	(0:1)	(1:1)	Selection effect, fix allele	347
3	(1:1) or (1:0)	(0:0)	Selection effect, allele loss	3520
4	(0:0)	(1:1)	New mutation, Fix allele	56
5	(0:0)	(0:1)	New mutation, partially fix allele	2716

tion of genes in this species is not sufficiently advanced (26% of genes with putative function [46]) and needs improvement.

The ratio of new and lost alleles (Table 3, ratio between the sum of entries 4 and 5, and entry 3) during the selection procedure is 0.79, which shows a selection effect appearing, weakly superior to the standard mutation rate. As suggested by these results, had the selection procedure been prolonged, an erosion of the genetic diversity would have occurred. This side-effect is commonly undesirable for the obtention of a non-clonal adaptive strain.

The genomic analysis also confirmed that more than one gene was modified. We then investigated whether the presence of polymorphisms in certain genes involved in carbon metabolism could explain the observed phenotype. However, genomic knowledge is limited for this species and among the genes studied (see supplementary information), we could not specifically identify genes and/or polymorphisms that clearly explain the phenotypic trait obtained. Thus, this genomic comparison only allows us to measure the impact of the improvement program on the genome of the improved strain, but in no case to propose genes likely to be involved in the phenotype obtained.

3.3. Final comparison between selected strains, S2X and the original strain, W2X

Results of the final batch experiment carried out to compare growth and biomass productivity of the W2X and S2X strains are represented in Fig. 2. As expected, both growth rate and cell density in S2X cultures were higher, eventually resulting in a twofold cell density and a carbon productivity increase of 77% at day 13 (Fig. 3). Cell density first grew exponentially (phase I) and then linearly, characterizing a photolimitation growth (phase II) [51]. Eventually, it saturated. Since irradiance (130 μmol quanta.m^2.s^{-1}) was lower than the I_{opt} value measured for the W2X strain [52], cells were most probably not photoinhibited.

The improved biomass productivity of S2X strain resulted from the combination of three possible causes. First, the continuous selection process (see Section 3.1) favored the emergence of cells with higher growth rates [29]. Second, as demonstrated by the benchmark experiment, the S2X strain is less rich in photosynthetic pigments for a similar light intensity. Consequently, a higher level of light is available in the reactor, therefore supporting a higher average growth rate of the population. Finally, the dynamical continuous selection process likely favored cells with an increased photoacclimation capacity allowing them to rapidly adapt their pigment content more efficiently to a change of photon flux density. Indeed, Bonnefond et al. [39] showed that a faster acclimation enhances growth and biomass productivity both under high and low light regimes.

The pigment evolution during the comparison experiment is represented in Fig. 4. Samples were taken simultaneously in batch reactors, and the population was systematically twice as large for S2X than W2X

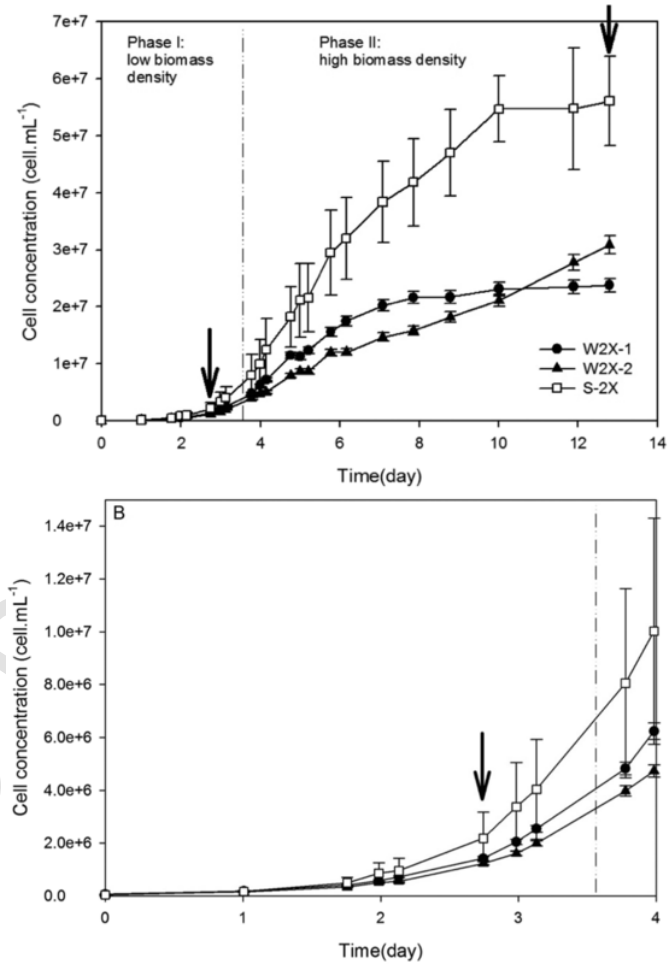

Fig. 2. A: cell concentration measured in final batch cultures of initial W2X and adapted S2X strains performed at the end of the selection experiment. Cell concentration measurement was performed in triplicates. W2X strain was grown in biological duplicate (W2X-1 and W2X-2, $n = 2$) and S2X strain in biological triplicates ($n = 3$). B: zoom of fig. A for the first four days. Black arrows represent the time of pigment sampling (see Fig. 4).

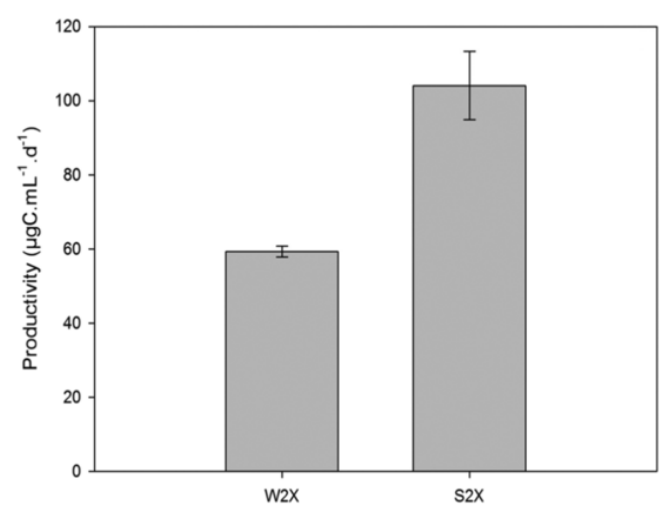

Fig. 3. Total carbon productivity of initial W2X and adapted S2X strains measured in batch cultures (see Fig. 2A). W2X strain productivity was measured in biological duplicate on triplicate measurements (n = 2) and S2X strain in biological triplicates on triplicate measurements (n = 3).

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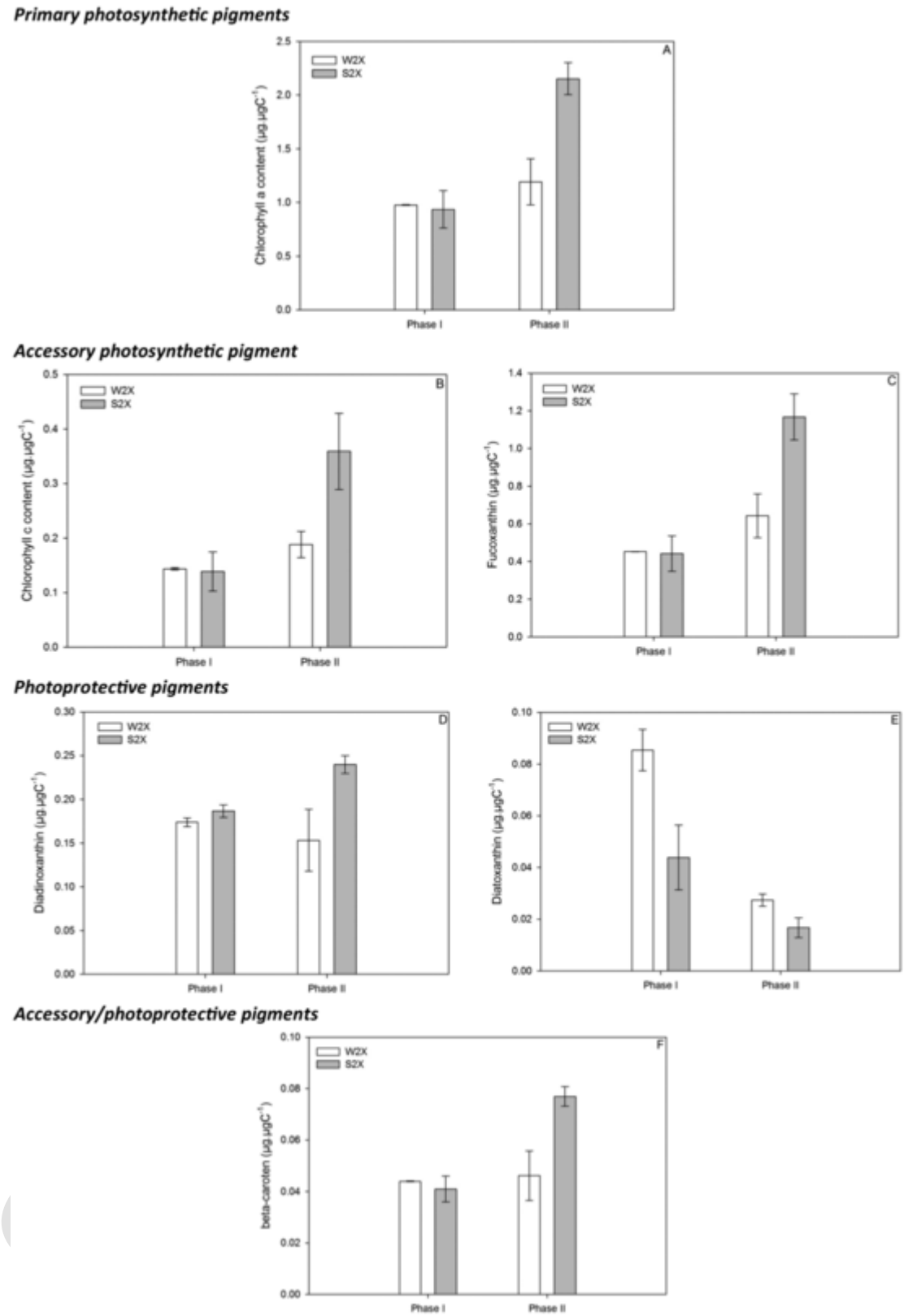

Fig. 4. Pigment content (A: chlorophyll *a*; B: chlorophyll *c*; C: fucoxanthin; D: diadinoxanthin; E: diatoxanthin; F: beta-carotene) measured in the initial W2X and adapted S2X strains during the phases I and II of final batch cultures (see Fig. 2A for sampling time). W2X strain pigment content was measured in biological duplicate on triplicate measurements (n = 2) and S2X strain in biological triplicates on triplicate measurements (n = 3).

cultures at both sampling times (Fig. 2). Therefore, available light was lower in S2X cultures even though cells had a lower pigment content. Consequently, pigment changes are a complex interplay between different light conditions and photoacclimation dynamics, and definitive conclusions must be made with care when comparing both strains' pigment content.

Despite these distinct conditions, at the time of pigment sampling during phase I, photosynthetic cell pigments (*i.e.*, chlorophyll *a* and *c* and fucoxanthin) are similar in both W2X and S2X strains (~0.90 μgChl*a*.μgC^{-1}, ~0.15 μgChl*c*.μgC^{-1} and ~ 0.40 μgFuc.μgC^{-1}, Fig. 4A, B and C respectively). Interestingly, albeit a lower available light and similar photosynthetic pigment content, S2X exhibited a significantly higher division rate than the W2X strain (1.45 d^{-1} ± 2.87 × 10^{-2}, p-value <0.001 and 1.16 d^{-1} ± 2.79 × 10^{-2}, p-value <0.001 respectively). By the end of phase II, light-limitation was more pronounced due to high biomass concentration and self-shading effects, particularly in S2X cultures. Light-harvesting pigment contents increased in both strains as an expected consequence of photolimitation [8,10]. The growth rate in phase II remained higher for the S2X strain (0.23 d^{-1} ± 2.87 × 10^{-2}, p-value <0.001 and 0.19 d^{-1} ± 2.95 × 10^{-2}, p-value <0.001, respectively), despite the twofold-higher cell density, which was expected to reduce available light and hence decrease growth rate more drastically. At this sampling time, cells in the S2X reactor have more chlorophyll *a* and *c*, further illustrating the different light and photoacclimation conditions (see Fig. 2).

The dynamics of the photoprotective pigments follow a similar logic. Diatoxanthin (Dt) and diadinoxanthin (Dd) are involved in the so-called xanthophyll cycle, consisting of one de-epoxidation step that converts Dd into Dt. Dt interacts with antenna complexes to convert the PSII light-harvesting complexes (LHC) antenna into a heat-dissipating state. Xanthophyll cycle-related energy dissipation (also known as non-photochemical quenching-NPQ) is thought to be a strategy to balance energy availability (absorbed light) and demand for photosynthates (growth) [53]. At higher irradiance (here in phase I, and especially for W2X), the excess excitation of PSII is generally accompanied by an increase in Dt and a decrease in Dd. Conversely, under light-limiting conditions, Dd accumulation *via* epoxidation has been reported [54]. Diatoxanthin decreased while irradiance was getting lower (Dt, Fig. 4E), favoring diadinoxanthin (Dd, Fig. 4D). This decrease was more pronounced in S2X cultures with double the biomass density. Dynamics might also show a better photoacclimation mechanism for the S2X population (Fig. 4E, phase I), which allowed a faster and efficient adjustment of the absorbed energy to the cell needs.

4. Conclusion

By applying a dynamical tailor-made selection pressure and exploiting the acclimation capability of *Tisochrysis lutea*, we managed to design a 77% more biomass productive microalgae strain successfully.

More data are needed to assess the stability of the selected strain when cultivated under non-stressing conditions for several months. Maintaining the selection pressure can be a strategy to preserve the metabolic changes, albeit prohibitive and cumbersome. Alternatively, the selected population may be screened to isolate clones with the desired phenotype. This additional step will prevent individuals in minority to outcompete the strain of interest [55]. Lastly, cryopreserving the selected microalgae will guarantee the conservation of the sought-after properties when inoculating a new reactor. Our work profoundly modifies the vision of previous GMO-based approaches and proposes an alternative perspective for solving the puzzle that photolimitation within microalgae cultures represents. Pigment deprived strains were obtained, but on top of improving the available light in the reactor, their photo-acclimation capacity was also likely enhanced, providing an additional, unexpected advantage. The high potential of selection pressures based on nature's capability to adapt was again [5] validated.

CRediT authorship contribution statement

Dr. Hubert Bonnefond and Yann Lie conceptualized and performed the selection experiments and methodology and wrote the corresponding sections of the manuscript.

Dr. Thomas Lacour, Dr. Benoit Saint-Jean and Dr. Gregory Carrier conceptualized, performed and designed the genomic analysis.

Amélie Talec conceptualized and performed the comparison experiment.

Éric Pruvost conceptualized and designed the photobioreactors.

Dr. Olivier Bernard and Dr. Antoine Sciandra were responsible for supervision/administration of the project, reviewing/editing of the manuscript and conceptualization of the selection and comparison experiments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] R.H. Wijffels, M.J. Barbosa, M.H.M. Eppink, Microalgae for the production of bulk chemicals and biofuels, Biofuels Bioprod. Biorefin. 4 (2010) 287–295, https://doi.org/10.1002/bbb.215.

[2] G. Carrier, M. Garnier, L. Le Cunff, G. Bougaran, I. Probert, C. De Vargas, E. Corre, J.-P. Cadoret, B. Saint-Jean, Comparative transcriptome of wild type and selected strains of the microalgae tisochrysis lutea provides insights into the genetic basis, lipid metabolism and the life cycle, PLoS One 9 (2014) e86889, https://doi.org/10.1371/journal.pone.0086889.

[3] L. Lardon, A. Hélias, B. Sialve, J.P. Steyer, O. Bernard, Life-cycle assessment of biodiesel production from microalgae, Environ. Sci. Technol. 43 (2009) 6475–6481, https://doi.org/10.1021/es900705j.

[4] P.J.le B. Williams, L.M.L. Laurens, Microalgae as biodiesel & biomass feedstocks: review & analysis of the biochemistry, energetics & economics, Energy Environ. Sci. 3 (2010) 554, https://doi.org/10.1039/b924978h.

[5] A. Nikolaou, P. Hartmann, A. Sciandra, B. Chachuat, O. Bernard, Dynamic coupling of photoacclimation and photoinhibition in a model of microalgae growth, J. Theor. Biol. 390 (2016) 61–72, https://doi.org/10.1016/j.jtbi.2015.11.004.

[6] E. Molina Grima, J.M. Fernández Sevilla, J.A. Sánchez Pérez, F. García Camacho, A study on simultaneous photolimitation and photoinhibition in dense microalgal cultures taking into account incident and averaged irradiances, J. Biotechnol. 45 (1996) 59–69, https://doi.org/10.1016/0168-1656(95)00144-1.

[7] P.G. Falkowski, Primary Productivity in the Sea, Springer US, Boston, MA, 1980, https://doi.org/10.1007/978-1-4684-3890-1.

[8] H.L. MacIntyre, T.M. Kana, T. Anning, R.J. Geider, Photoacclimation of photosynthesis irradiance response curves and photosynthetic pigments in microalgae and cyanobacteria, J. Phycol. 38 (2002) 17–38, https://doi.org/10.1046/j.1529-8817.2002.00094.x.

[9] K. Richardson, J. Beardall, J.A. Raven, Adaptation of unicellular algae to irradiance: an analysis of strategies, New Phytol. 93 (1983) 157–191, https://doi.org/10.1111/j.1469-8137.1983.tb03422.x.

[10] M.R. Brown, G.A. Dunstan, S.W. Jeffrey, J.K. Volkman, S.M. Barrett, J.-M. LeRoi, The influence of irradiance on the biochemical composition of the prymnesiophyte isochrysis sp. (Clone T-Iso), J. Phycol. 29 (1993) 601–612, https://doi.org/10.1111/j.0022-3646.1993.00601.x.

[11] T. Anning, H.L. MacIntyre, S.M. Pratt, P.J. Sammes, S. Gibb, R.J. Geider, Photoacclimation in the marine diatom Skeletonema costatum, Limnol. Oceanogr. 45 (2000) 1807–1817, https://doi.org/10.4319/lo.2000.45.8.1807.

[12] H. Kirst, J.G. Garcia-Cerdan, A. Zurbriggen, T. Ruehle, A. Melis, Truncated photosystem chlorophyll antenna size in the green microalga Chlamydomonas reinhardtii upon deletion of the TLA3-CpSRP43 gene, Plant Physiol. 160 (2012) 2251–2260, https://doi.org/10.1104/pp.112.206672.

[13] A. Melis, J. Neidhardt, J.R. Benemann, Dunaliella Salina (Chlorophyta) with

UNCORRECTED PROOF

small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells, J. Appl. Phycol. 10 (1998) 515–525, https://doi.org/10.1023/A:1008076231267.
[14] J. Neidhardt, J.R. Benemann, L. Zhang, A. Melis, Photosystem-II repair and chloroplast recovery from irradiance stress: relationship between chronic photoinhibition, light-harvesting chlorophyll antenna size and photosynthetic productivity in Dunaliella Salina (green algae), Photosynth. Res. 56 (1998) 175–184, https://doi.org/10.1023/A:1006024827225.
[15] B.M. Smith, P.J. Morrissey, J.E. Guenther, J.A. Nemson, M.A. Harrison, J.F. Allen, A. Melis, Response of the Photosynthetic Apparatus in Dunaliella salina (Green Algae) to Irradiance Stress, Plant Physiol. 93 (1990) 1433–1440, https://doi.org/10.1104/pp.93.4.1433.
[16] M. Willemoës, E. Monas, Relationship between growth irradiance and the xanthophyll cycle pool in the diatom Nitzschia Palea, Physiol. Plant. 83 (1991) 449–456, https://doi.org/10.1111/j.1399-3054.1991.tb00119.x.
[17] T. de Mooij, M. Janssen, O. Cerezo-Chinarro, J.H. Mussgnug, O. Kruse, M. Ballottari, R. Bassi, S. Bujaldon, F.-A. Wollman, R.H. Wijffels, Antenna size reduction as a strategy to increase biomass productivity: a great potential not yet realized, J. Appl. Phycol. 27 (2015) 1063–1077, https://doi.org/10.1007/s10811-014-0427-y.
[18] A. Melis, Solar energy conversion efficiencies in photosynthesis: minimizing the chlorophyll antennae to maximize efficiency, Plant Sci. 177 (2009) 272–280, https://doi.org/10.1016/j.plantsci.2009.06.005.
[19] M.H. Huesemann, T.S. Hausmann, R. Bartha, M. Aksoy, J.C. Weissman, J.R. Benemann, Biomass productivities in wild type and pigment mutant of cyclotella sp. (Diatom), Appl. Biochem. Biotechnol. 157 (2009) 507–526, https://doi.org/10.1007/s12010-008-8298-9.
[20] Y. Nakajima, R. Ueda, Improvement of photosynthesis in dense microalgal suspension by reduction of light harvesting pigments, J. Appl. Phycol. 9 (1997) 503–510, https://doi.org/10.1023/A:1007920025419.
[21] G. Perin, A. Bellan, A. Segalla, A. Meneghesso, A. Alboresi, T. Morosinotto, Generation of random mutants to improve light-use efficiency of nannochloropsis gaditana cultures for biofuel production, Biotechnol. Biofuels 8 (2015) 161, https://doi.org/10.1186/s13068-015-0337-5.
[22] J.E.W. Polle, S.-D. Kanakagiri, A. Melis, tla1, a DNA insertional transformant of the green alga Chlamydomonas reinhardtii with a truncated light-harvesting chlorophyll antenna size, Planta 217 (2003) 49–59, https://doi.org/10.1007/s00425-002-0968-1.
[23] S. Cazzaniga, L. Dall'Osto, J. Szaub, L. Scibilia, M. Ballottari, S. Purton, R. Bassi, Domestication of the green alga Chlorella sorokiniana: reduction of antenna size improves light-use efficiency in a photobioreactor, Biotechnol. Biofuels 7 (2014) 157, https://doi.org/10.1186/s13068-014-0157-z.
[24] Y. Nakajima, R. Ueda, The effect of reducing light-harvesting pigment on marine microalgal productivity, J. Appl. Phycol. 12 (2000) 285–290, https://doi.org/10.1023/A:1008108500409.
[25] J.H. Mussgnug, S. Thomas-Hall, J. Rupprecht, A. Foo, V. Klassen, A. McDowall, P.M. Schenk, O. Kruse, B. Hankamer, Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion, Plant Biotechnol. J. 5 (2007) 802–814, https://doi.org/10.1111/j.1467-7652.2007.00285.x.
[26] H. Kirst, J.G. García-Cerdán, A. Zurbriggen, A. Melis, Assembly of the light-harvesting chlorophyll antenna in the green alga Chlamydomonas reinhardtii requires expression of the TLA2 - CpFTSY gene, Plant Physiol. 158 (2012) 930–945, https://doi.org/10.1104/pp.111.189910.
[27] G. Bonente, C. Formighieri, M. Mantelli, C. Catalanotti, G. Giuliano, T. Morosinotto, R. Bassi, Mutagenesis and phenotypic selection as a strategy toward domestication of Chlamydomonas reinhardtii strains for improved performance in photobioreactors, Photosynth. Res. 108 (2011) 107–120, https://doi.org/10.1007/s11120-011-9660-2.
[28] G. Hardin, The competitive exclusion principle, Science (80-.) 131 (1960) 1292–1297, https://doi.org/10.1126/science.131.3409.1292.
[29] P. Masci, O. Bernard, F. Grognard, P. Masci, O. Bernard, F. Grognard, C. Selection, F. Growing, P. Masci, O. Bernard, Continuous selection of the fastest growing species in the chemostat to cite this version : HAL id : hal-01091821 continuous selection of the fastest growing species in the chemostat, IFAC Proc. 85 (2008).
[30] C. Combe, P. Hartmann, S. Rabouille, A. Talec, O. Bernard, A. Sciandra, Long-term adaptive response to high-frequency light signals in the unicellular photosynthetic eukaryote Dunaliella Salina, Biotechnol. Bioeng. 112 (2015) 1111–1121, https://doi.org/10.1002/bit.25526.
[31] M. Gachelin, M. Boutoute, G. Carrier, A. Talec, E. Pruvost, F. Guihéneuf, O. Bernard, A. Sciandra, Enhancing PUFA-rich polar lipids in tisochrysis lutea using adaptive laboratory evolution (ALE) with oscillating thermal stress, Appl. Microbiol. Biotechnol. 105 (2021) 301–312, https://doi.org/10.1007/s00253-020-11000-4.
[32] X. Reboud, N. Majerus, J. Gasquez, S. Powles, Chlamydomonas reinhardtii as a model system for pro-active herbicide resistance evolution research, Biol. J. Linn. Soc. 91 (2007) 257–266, https://doi.org/10.1111/j.1095-8312.2007.00787.x.
[33] B. Rico-Villa, J.R. Le Coz, C. Mingant, R. Robert, Influence of phytoplankton diet mixtures on microalgae consumption, larval development and settlement of the Pacific oyster Crassostrea gigas (Thunberg), Aquaculture 256 (2006) 377–388, https://doi.org/10.1016/j.aquaculture.2006.02.015.
[34] P.A. Thompson, M. Guo, P.J. Harrison, J.N.C. Whyte, Effects of variation in temperature. II. On the fatty acid composition of eight species of marine phytoplankton, J. Phycol. 28 (1992) 488–497, https://doi.org/10.1111/j.0022-3646.1992.00488.x.
[35] S.M. Renaud, H.C. Zhou, D.L. Parry, L.-V. Thinh, K.C. Woo, Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae isochrysis sp., Nitzschia closterium, Nitzschia paleacea, and commercial species isochrysis sp. (clone T.ISO), J. Appl. Phycol. 7 (1995) 595–602, https://doi.org/10.1007/BF00003948.
[36] J. Liu, M. Sommerfeld, Q. Hu, Screening and characterization of isochrysis strains and optimization of culture conditions for docosahexaenoic acid production, Appl. Microbiol. Biotechnol. 97 (2013) 4785–4798, https://doi.org/10.1007/s00253-013-4749-5.
[37] G. Bougaran, C. Rouxel, N. Dubois, R. Kaas, S. Grouas, E. Lukomska, J.-R. Le Coz, J.-P. Cadoret, Enhancement of neutral lipid productivity in the microalga Isochrysis affinis galbana (T-Iso) by a mutation-selection procedure, Biotechnol. Bioeng. 109 (2012) 2737–2745, https://doi.org/10.1002/bit.24560.
[38] D. Padfield, Genevieve Yvon-Durocher, A. Buckling, S. Jennings, Gabriel Yvon-Durocher, Rapid evolution of metabolic traits explains thermal adaptation in phytoplankton, Ecol. Lett. 19 (2016) 133–142, https://doi.org/10.1111/ele.12545.
[39] H. Bonnefond, G. Grimaud, J. Rumin, G. Bougaran, A. Talec, M. Gachelin, M. Boutoute, E. Pruvost, O. Bernard, A. Sciandra, Continuous selection pressure to improve temperature acclimation of tisochrysis lutea, PLoS One 12 (2017) e0183547, https://doi.org/10.1371/journal.pone.0183547.
[40] R.R.L. Guillard, J.H. Ryther, Studies of marine planktonic diatoms. I. Cyclotella nana hustedt, and Detonula confervacea (CLEVE) gran, Can. J. Microbiol. 8 (1962) 229–239.
[41] O. Bernard, Odin [WWW document], URL. https://team.inria.fr/biocore/software/odin/, 2016. (accessed 8.5.20).
[42] H. Bonnefond, N. Moelants, A. Talec, O. Bernard, A. Sciandra, Concomitant effects of light and temperature diel variations on the growth rate and lipid production of Dunaliella Salina, Algal Res. 14 (2016) 72–78, https://doi.org/10.1016/j.algal.2015.12.018.
[43] D. Stramski, A. Sciandra, H. Claustre, Effects of temperature, nitrogen, and light limitation on the optical properties of the marine diatom Thalassiosira pseudonana, Limnol. Oceanogr. 47 (2002) 392–403, https://doi.org/10.4319/lo.2002.47.2.0392.
[44] Z. Hu, X. Zeng, A. Wang, C. Shi, D. Duan, An efficient method for DNA isolation from red algae, J. Appl. Phycol. 16 (2004) 161–166, https://doi.org/10.1023/B:JAPH.0000048456.26639.1a.
[45] M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads, EMBnet.journal 17 (2011) 10, https://doi.org/10.14806/ej.17.1.200.
[46] J. Berthelier, N. Casse, N. Daccord, V. Jamilloux, B. Saint-Jean, G. Carrier, A transposable element annotation pipeline and expression analysis reveal potentially active elements in the microalga tisochrysis lutea, BMC Genomics 19 (2018) 378, https://doi.org/10.1186/s12864-018-4763-1.
[47] H. Li, R. Durbin, Fast and accurate long-read alignment with burrows-wheeler transform, Bioinformatics 26 (2010) 589–595, https://doi.org/10.1093/bioinformatics/btp698.
[48] E. Garrison, G. Marth, in: Haplotype-based Variant Detection From Short-read Sequencing, 2012, pp. 1–9.
[49] A. Dereeper, F. Homa, G. Andres, G. Sempere, G. Sarah, Y. Hueber, J.-F. Dufayard, M. Ruiz, SNiPlay3: a web-based application for exploration and large scale analyses of genomic variations, Nucleic Acids Res. 43 (2015), https://doi.org/10.1093/nar/gkv351, W295-W300.
[50] E. Le Floc'h, G. Malara, A. Sciandra, An automatic device for in vivo absorption spectra acquisition and chlorophyll estimation in phytoplankton cultures, J. Appl. Phycol. 14 (2002) 435–444, https://doi.org/10.1023/A:1022338930747.
[51] I. Wagner, C. Steinweg, C. Posten, Mono- and dichromatic LED illumination leads to enhanced growth and energy conversion for high-efficiency cultivation of microalgae for application in space, Biotechnol. J. 11 (2016) 1060–1071, https://doi.org/10.1002/biot.201500357.
[52] J. Marchetti, G. Bougaran, L. Le Dean, C. Mégrier, E. Lukomska, R. Kaas, E. Olivo, R. Baron, R. Robert, J.P. Cadoret, Optimizing conditions for the continuous culture of Isochrysis affinis galbana relevant to commercial hatcheries, Aquaculture 326–329 (2012) 106–115, https://doi.org/10.1016/j.aquaculture.2011.11.020.
[53] T. Lacour, M. Babin, J. Lavaud, Diversity in xanthophyll cycle pigments content and related nonphotochemical quenching (NPQ) among microalgae: implications for growth strategy and ecology, J. Phycol. 56 (2020) 245–263, https://doi.org/10.1111/jpy.12944.
[54] M. Lohr, C. Wilhelm, Algae displaying the diadinoxanthin cycle also possess the violaxanthin cycle, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 8784–8789, https://doi.org/10.1073/pnas.96.15.8784.
[55] F. Gao, M. Sá, I.T.D. Cabanelas, R.H. Wijffels, M.J. Barbosa, Improved fucoxanthin and docosahexaenoic acid productivities of a sorted self-settling tisochrysis lutea phenotype at pilot scale, Bioresour. Technol. 325 (2021) 124725, https://doi.org/10.1016/j.biortech.2021.124725.

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