Title: Effect of temperature on growth, photosynthesis and biochemical composition of *Nannochloropsis oceanica,* grown outdoors in tubular photobioreactors

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Abstract (300 words)

Since temperature is an important factor affecting microalgal growth, photosynthetic rate and biomass composition, this study has accordingly focused on its effects on biomass yield and nighttime biomass loss, as well as photochemical changes, using *Nannochloropsis oceanica* as model species, grown in two outdoor 50-L tubular photobioreactors (PBR). In two independent trials, cultures were subjected to a diurnal light:dark cycle, under a constant temperature of 28 ºC and, on the second trial, at 18 ºC. Changes in culture performance were assessed by measuring growth and assessing lipid and fatty acid composition of the biomass in both morning and evening. Our results revealed that *N. oceanica* shows a wide temperature tolerance with relevant nighttime biomass loss, that decreased at lower

temperatures, at the expenses of its daily productivity. Fluorescence measurements revealed reversible damage to photosystem II in cells growing in the PBR under optimal thermal conditions, whereas microalgae grown at suboptimal ones exhibited an overall lower photosynthetic activity. Lipids were partially consumed overnight to support cell division and provide maintenance energy. Eicosapentaenoic acid (EPA) catabolism reached a maximum after the dark period, as opposed to their saturated counterparts; whereas lower temperatures led to higher EPA content which reached the maximum in the morning. These findings are relevant for the production of *Nannochloropsis* at industrial scale.

Keywords (max 6 keywords):

Nannochloropsis, temperature, photosynthesis, respiration, night biomass loss, chlorophyll fluorescence.

1. Introduction

Microalgae entail a multitude of applications that make them attractive as commercial commodities [1]. However, the industrial production of microalgal biomass is hindered by many biotic and abiotic factors that reduce their productivity, thus compromising attempts to reduce production costs. Suboptimal temperature is one of the environmental factors known to significantly decrease biomass productivity [2,3]. Unfavourable thermal conditions can bring about a decrease in biomass productivity throughout the day, as well as an increased nighttime biomass loss [3,4]. In fact, cells metabolize a wide array of reserve biomolecules at night, in order to produce maintenance energy, support cell division, and provide carbon skeletons for the synthesis of compounds, resulting in biomass loss [5]. Previous reports have described nighttime biomass losses reaching up to 30% of the daily yield, and the highest value reported was 42% of the daily yield by *Arthrospira platensis* [6–8]. In addition, microalgae are

exposed to high radiation levels when grown outdoors which, combined with suboptimal temperatures, can unbalance biochemical composition and promote nighttime biomass losses [5]. Since both temperature and irradiance have a great influence on photosynthesis, and concomitant biomass growth, it is important that these parameters are monitored for putative commercial applications – and, if possible, adjusted to their optimal values, making it possible to direct synthesis towards specific target molecules.

The Eustigmatophyte *Nannochloropsis oceanica* is a unicellular, fast growing, resilient microalga, able to accumulate large quantities of lipids and eicosapentaenoic acid (EPA), which constitutes a key factor for its commercial development [9]. The aim of this study was to study the influence of suboptimal temperature on the growth physiology, biochemical composition and nighttime biomass loss of *N. oceanica*, grown outdoors in thermoregulated tubular photobioreactors (PBRs).

2. Material and methods

2.1. Outdoor pilot PBR

The study was carried out in Florence, Italy (N 43.8 º, E 11.3 º). Each PBR consisted of ten parallel Pyrex tubes (length 2 m, i.d. 4.85 cm) connected by PVC (polyvinylchloride) U-bends with watertight flanges, providing a working volume of 50 L. Each reactor was placed in a stainless-steel basin containing thermostated demineralized water. The culture was recycled by a PVC pump having three flat PVC blades at 120 º to each other on the propeller shaft; the distance between blades and casing was 1.3 cm. The PBR included a 2.2-L transparent PVC cylindrical degasser where air and CO_2 were supplied. The cultures were operated at a constant pH of 8, by CO_2 injection on demand. A circadian cycle was set by covering the cultures with a dark plastic sheet in the evening at 6:00 pm and removing it at 8:00 am of the following morning, thus providing a 10:14 light:dark (L:D) period. Two identical tubular PBRs were used for the experiments.

2.2. Experimental set-up

One PBR (i.e. the control) was kept at 28 ± 1 ºC during the light periods, from 8:00 am to 6:00 pm; in the evening, the temperature was decreased to 18 ± 1 ºC and kept constant overnight (from 6:00 pm to 8:00 am). Two sequential experiments were carried out, in which two different temperature regimes were applied to the second PBR (Table 1). In the first trial, the PBR was kept at the constant temperature of 28 ± 1 ºC by day and by night. While in the second trial, the PBR was maintained at 18 ºC ± 1 ºC by day and by night.

Table 1 Experimental plan reporting the temperatures during daytime (starting at 8:00 am) and during nighttime (starting at 6:00 pm) for each condition within each trial. Because only two photobioreactors (PBR) were available, the trials were carried out in sequence with a control PBR for each experiment.

Trial	Condition	Daylight temperature (8 am-6 pm)	Nighttime temperature (6 pm-8 am)	Experimental period	Mean daily irradiance (MJ m^{-2} day^{-1} ± SD)
1	28:18 °C L:D	28 ± 1 ºC	18 ± 1 ºC	July 26th to August 3rd	20.3 ± 3.21
	28:28 °C L:D	28 ± 1 ºC	28 ± 1 ºC		
2	28:18 °C L:D	28 ± 1 ºC	18 ± 1 ºC	August 27 - September 4	16.4 ± 4.03
	18:18 °C L:D	18 ± 1 ºC	18 ± 1 ºC		

2.3. Microorganism and culture conditions

The microalga *N. oceanica*, kindly provided by Dr. Avigad Vonshak (Institute for Desert Research, Ben-Gurion University, Israel), was selected for its fast growth, high lipid content and commercial interest. Cultivation was scaled up to two outdoor 50-L tubular PBRs using F medium enriched with $NaHCO_3$ (6 mM), and 10-fold the values of $NaNO_3$ (17.7 mM) and NaH_2PO_4 (0.84 mM) of the original recipe [10]. Once the culture reached a biomass concentration of ca. 0.8 g L^{-1} of dry weight (DW), a semi-continuous regime was initiated. For this purpose, a part of the culture was removed every day and replaced with fresh medium to achieve ca. 0.45 g L^{-1} of DW at the beginning of the light period, which avoided any nutrient depletion. Solar irradiance data were supplied by the Laboratory for Meteorology and Environmental Modelling (LAMMA; CNR, Florence, Italy), located close to the area where experiments took place.

Sampling was performed every morning at 8:00 am (i.e., before removing the culture covers), and at 6:00 pm at the end of light period (i.e., before covering the PBRs).

2.4. Measurements

2.4.1. Growth

Growth was assessed through both biomass dry weight (DW) and cellular concentration. DW measurements were performed in duplicate by filtering 10 mL of culture through pre-weighed 47-mm diameter glass microfiber filter membranes (Whatman GF/F, Maidstone, England). The pellets on the filters were washed twice with deionized water and oven-dried afterwards at 105 ºC until constant weight. Cell counts were carried out in duplicate, using a Bürker counting chamber. Nighttime biomass loss was calculated as the difference between DW measured in the evening and that measured in the following morning just before removing the covers.

2.4.2. Fluorescence measurements

Chlorophyll fluorescence measurements were carried out with a pulse-amplitude-modulation fluorimeter (PAM-2100, H. Walz, Effeltrich, Germany), operated with PamWin (version 2.00f) PC software. Triplicate samples were incubated for 15 min in the dark, to oxidize all plastoquinone (Q_A) and ensure membrane relaxation. A weak light (0.5 µmol m^{-2} s^{-1}) was applied to measure the minimum fluorescence in the dark-adapted state (F_0). A strong saturating pulse (6000 µmol m^{-2} s^{-1}) was then supplied to close all reaction centres (RC; i.e. to fully reduce all Q_A) and reach the maximum fluorescence yield (F_m). The maximum photochemical quantum yield of PSII (F_v/F_m) was then calculated as the ratio between variable ($F_v = F_m - F_0$) and maximum fluorescence (F_m) [11,12].

Kautsky's curves (OJIP fluorescence induction kinetics) were recorded daily, in the morning and evening, using the Handy Plant Efficiency Analyser (PEA; Hansatech Instruments). Samples were dark-adapted for 15 min, and then illuminated with continuous saturating light. The fluorescence

transients were analysed with the Biolyzer HP3 software package. Since the light intensities were high enough to reach the maximum value F_m, the transients were normalized both on F_0 and F_m basis, so as to permit the best comparison of the shapes of the curves. Measurements were conducted in triplicate. OJIP-determined parameters for each condition were normalized to their corresponding controls, in order to clarify the impacts of temperature upon the photosynthetic machinery. OJIP curves are shown for the second and seventh day (which is the last complete day) of each trial.

2.4.3. Oxygen evolution and respiration measurements

For photosynthetic oxygen evolution measurements, 2 mL samples were loaded in a Liquid-Phase Oxygen Electrode Chamber (Hansatech, DW3) thermostated at 28 ºC, and equipped with an oxygen control electrode unit (Hansatech, Oxy-lab) and a magnetic stirrer. Light was supplied via a red light-emitting diode (LED) source (Hansatech LH36/2R), with nominal wavelength of 660 nm, providing 600 μmol photons m^{-2} s^{-1}. Before oxygen measurements, samples were purged with N_2 to reduce dissolved O_2 below saturation. Afterwards, the light was turned on, and the O_2 concentration monitored at an acquisition frequency of 1 reading s^{-1}. Measurements of respiration were carried out in triplicate, at the end of the photosynthesis measurements. Results are shown in μmol of O_2 μg^{-1} chl *a* h^{-1}.

2.4.4. Pigments

For pigment extraction, duplicate samples of 2 mL were collected, and centrifuged at 2650 *g* for 10 min. The supernatant was discarded, and glass beads were added to the pellet – which was then resuspended in 3 mL of acetone (90% v/v). The samples were vortexed for 5 min and centrifuged again. The supernatant was transferred to a clean tube, and the pellet subjected to two additional extractions with 2 mL of acetone, and a final centrifugation. Absorbance measurements were taken at 663 (A_{663}) and 750 nm (A_{750}, to correct for turbidity). The total chlorophyll *a* was determined based on the equation by SCOR-UNESCO (1966):

$$\text{Chlorophyll } a \ (\mu g \ mL^{-1}) = 11.64 \ (A_{663} - A_{750}) \ ; \quad (1)$$

the equation was truncated, since this genus is known to possess only chlorophyll *a* [13].

2.4.5. Lipid content

The lipid content was determined by collecting 5 mL of culture, in triplicate, which was centrifuged at 2650 *g* for 10 min. Lipids were extracted according to Bligh and Dyer (1959) [14], and quantified according to Marsh and Weinstein (1966) [15]. The pellets were previously washed with a solution of sodium chloride (9 g L^{-1}). For extraction, a mixture of chloroform:methanol (1:2 v/v) was used, along with bead beating for 5 min, and sample heating in a thermoblock at 60 ºC for 3 min. Following extraction, the organic phase was collected and dried. Extracts were resuspended in a known volume of chloroform and distributed in duplicate. The samples were heated in sulphuric acid at 200 ºC for 15 min, along with the prepared standard of tripalmitine (Sigma-Aldrich, USA). After the samples were cooled and distilled water duly added, the absorbance was read at 375 nm. Measurements are expressed in pg of lipids $cell^{-1}$.

2.4.6. Fatty acid analysis

Extraction and conversion of the samples to fatty acid methyl esters (FAME) were done following a protocol by Folch (1957) and Lepage and Roy (1984), with modifications as described by Pereira et al. (2012) [16–18]. Briefly, freeze-dried biomass samples were mixed in a reaction vessel with a solution of methanol:acetyl chloride (20:1 v/v), and then homogenized with an Ultra-Turrax disperser (1.5 min at 23000 rpm; T18 digital ULTRA-TURRAX, IKA-Werke GmbH & Co. KG, Staufen, Germany). After adding *n*-hexane, the mixture was subjected to derivatization at 70°C for 1 hr. The lipidic phase was separated via addition of distilled water and *n*-hexane, with vortexing and centrifuging samples (this step was repeated 3 times). The residual water of the organic phase was removed by adding anhydrous sodium sulphate. Extracts were filtered, dried under a nitrogen gas flow, and resuspended

in GC grade *n*-hexane. Analysis was performed in a Bruker gas chromatograph, coupled with MS detector (Bruker SCION 456/GC, SCION TQ MS) equipped with a ZB-5MS capillary column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness, Phenomenex), and using helium as carrier gas. The GC oven temperature profile was set to 60 ºC (1 min), 30 °C min^{-1} to 120 ºC, 5 °C min^{-1} to 250 ºC, and 20 ºC min^{-1} to 300 ºC (2 min). The commercial standard, Supelco® 37 Component FAME Mix (Sigma-Aldrich, Sintra, Portugal), was used to prepare the different calibration curves. Results are expressed as percentage of the total fatty acid content.

2.4.7. Statistical analysis

In each day, differences in biomass growth and loss between each condition (28:28 ºC L:D and 18:18 ºC L:D), relative to the corresponding controls, were analysed using repeated-measures ANOVA – followed by an assessment of linear relationships using Pearson's test ($p < 0.05$).

A one-way ANOVA, followed by Tukey's post-hoc test, was performed to detect statistical differences between conditions (28:28 ºC L:D and 18:18 ºC L:D) and the corresponding controls. All tests were done using R software. The significance level was set as $\alpha = 0.05$.

3. Results & Discussion

3.1. Growth

N. oceanica grown in a semi-continuous regime in tubular PBR outdoors (July to September of 2018), showed, in both trials, a clear biomass loss during the night period (see Figure 1). The night biomass loss pattern was mirrored by changes in cell number, which were maximal at the end of the dark period (data not shown). These findings are supported by previous reports that documented a timed cell division occurring during the dark periods in *Nannochloropsis* cells [19,20].

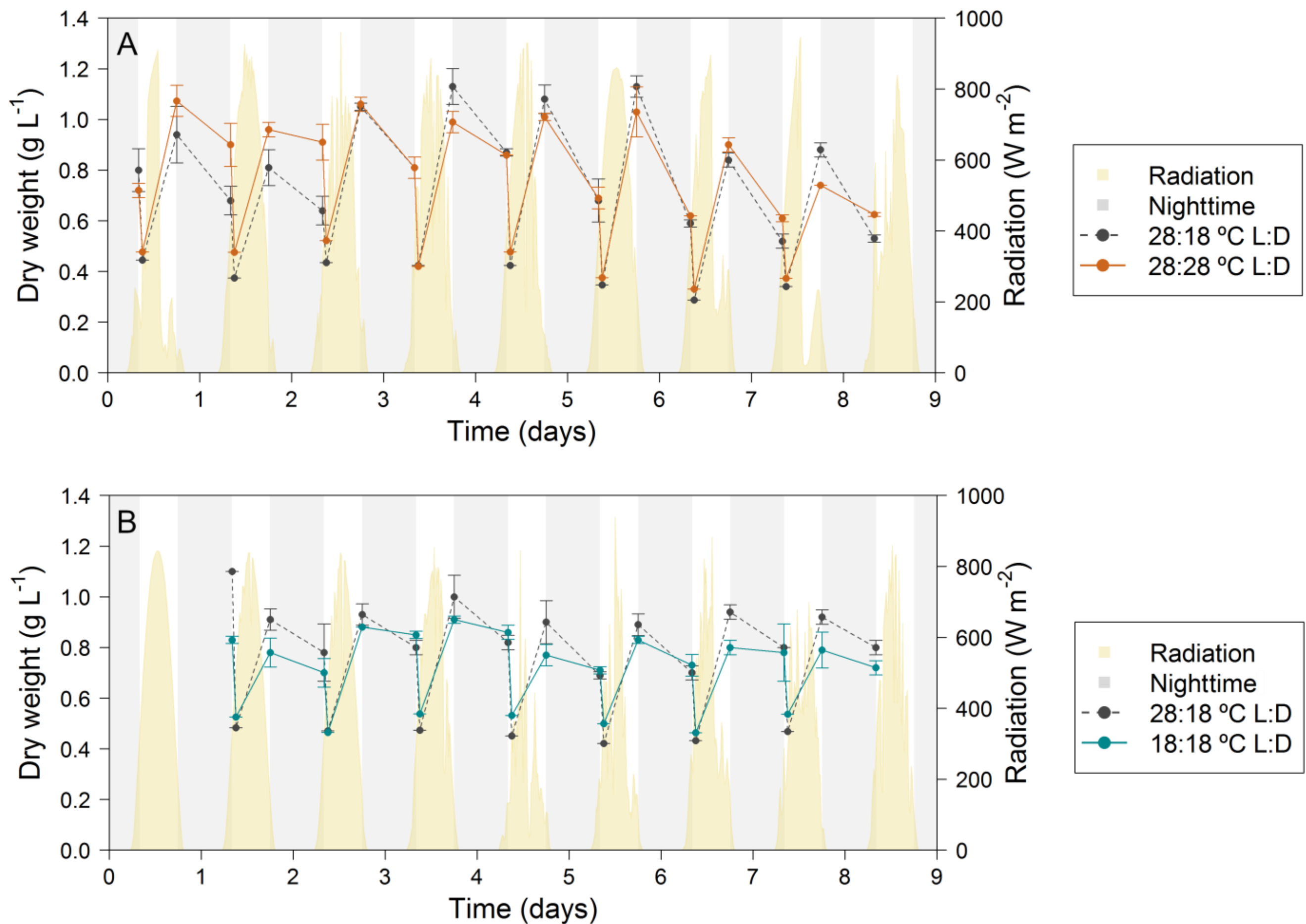

Figure 1 Changes in *Nannochloropsis oceanica* biomass dry weight (DW) grown outdoors in a semi-continuous mode under different temperature regimes(*n*=2). After the morning sample, cultures were diluted to 0.45 g L^{-1} of DW and allowed to grow until the following morning. Both the first (A) and second (B) trials show the respective 28:18 °C L:D controls (dashed grey lines), the programmed night periods when the photobioreactors were covered (light grey bars), and the solar irradiance for each day (yellow). Each trial shows the tested condition (28:28 °C L:D and 18:18 °C L:D), represented as a solid line in orange for the first trial (A) and in blue for the second trial (B).

The maximum dry weights in the first trial were 1.13±0.07 and 1.07±0.06 g L^{-1}, for the control culture maintained at 28:18 °C L:D and for the one kept at 28:28 °C L:D, respectively. While in the second trial, the culture maintained at 28:18 °C L:D reached 1.00±0.08, and the culture grown at 18:18 °C L:D reached 0.91±0.01 g L^{-1}.

In order to have a better grasp of the biomass variations due to temperature, each day was used as a replicate and the daily yield and biomass loss were compared (Figure 2). In both trials, performed in July-August and August-September, the cultures kept at dual temperatures (28:18 °C L:D), revealed higher daylight productivities, of 0.57±0.09 and 0.47±0.04 g L^{-1} day^{-1}, but also higher nighttime biomass losses, reaching up to 53% and 33% of the daily produced biomass in trial 1 and 2,

respectively. Differences between controls are a result of a slightly higher solar irradiation in the first trial when compared to trial 2, that took place between August and September, 2018, characterized by more unstable weather conditions. Detailed information on daily productivities and night losses can be found in Supplementary Table S1.

Figure 2 Daily average (+SD) of biomass dry weight (DW) changes in *Nannochloropsis oceanica* grown outdoors in a semi-continuous mode, under different temperature regimes, in two sequential trials (trial 1 *n*=8; trial 2 *n*=7). Positive and negative values represent the daily biomass yield and loss, respectively. Dots represent individual data points for each condition. The first two bars (Trial 1) represent the cells kept at 28:18 °C L:D (grey) and the ones kept at 28:18 °C L:D (orange). The last two bars (Trial 2) represent the cells kept at 28:18 °C L:D (grey) and the cells kept at 18:18 °C L:D (blue). Significant differences (p<0.05) to the respective control are marked with an asterisk (*).

The culture maintained at 28:28 ºC (L:D) produced a similar response to the control culture (28:18 ºC, L:D), although less pronounced for both productivity and nighttime loss. However, in other studies, this difference was more notorious. Upon using *N. salina*, higher biomass losses at higher temperatures in the dark were found [7]. A comparable outcome was also reported in previous experiments with *Chlorella pyrenoidosa*, *Phaeodactylum tricornutum* and *A. platensis,* in which lower nighttime temperatures led to lower biomass loss [4,5,21].

The slowest growth was achieved in cultures maintained at 18:18 ºC L:D, which can be considered as a suboptimal temperature for *Nannochloropsis* spp. growth. The optimum growth temperature for microalgae of this genus lies around 24-26 ºC, whereas values above 30 ºC or below 15-20 ºC are known to be supra- and suboptimal temperatures, respectively [22–30]. In the culture grown at the 18:18 ºC (L:D) temperature regime, both a lower daylight productivity (0.31 g L^{-1} day^{-1}) and night

biomass loss (as much as 19%) were found. An opposite behaviour was reported in a previous study using *C. pyrenoidosa*, where the night biomass loss increased as growth temperature decreased, although the lowest day temperature used was 25 ºC, which was significantly higher than the 18 ºC used in this study [5]. A slightly faster growth was previously found in *N. oculata*, kept under sinusoidally-varying temperatures, than at a fixed temperature [31]. Although different conclusions were reached using *Tetraselmis suecica* and cooling overnight [27], our findings suggest that the dual temperature regime allows the culture to recover faster after the high irradiances received during daytime, thus resulting in higher productivities, as seen in the cultures grown at 28:18 °C (L:D). However, the higher growth was coupled to a higher nighttime biomass loss. In order to find out whether this correlation held a statistical meaning, the productivities versus the corresponding nighttime biomass loss are graphically reported, combining every data point of both trials (Figure 3).

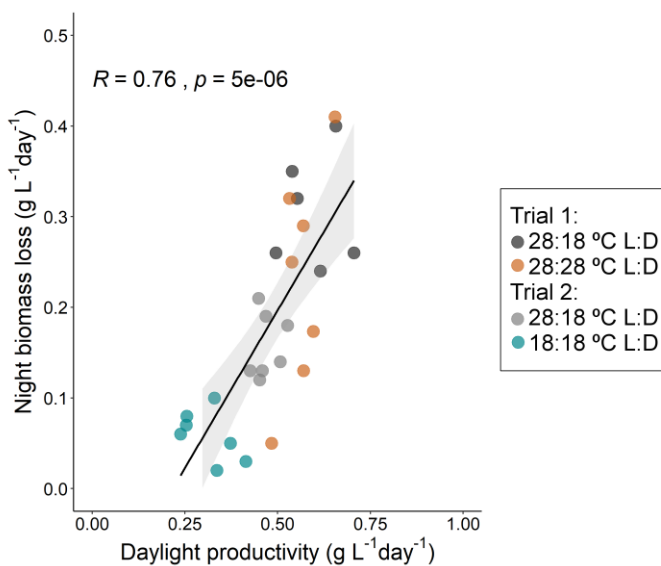

Figure 3 Correlation between daily yield and biomass loss, combining all data points from both trials using *Nannochloropsis oceanica* grown outdoors in tubular photobioreactors under different temperatures. Data points from the 28:18 ºC L:D (controls) cultures are depicted in dark (Trial 1) and light (Trial 2) grey; cells kept at 28:28 ºC L:D (Trial 1) and 18:18 ºC L:D (Trial 2) are depicted in orange and blue, respectively. The shaded grey area represents the confidence interval (95%) of Pearson's correlation. The correlation coefficient (R = 0.76) and the significance level ($p<0.05$) are shown in the upper left corner of the plot (n=27).

As can be grasped in Figure 3, the daily biomass yield (productivity) exhibited a positive correlation to the nighttime biomass loss ($R(25)$=0.76, p<0.05). Linear relationships between biomass loss and growth rates have been reported before in experiments encompassing nutrient-sufficient cultures kept

in L:D cycles [8,32]. The high dispersion of the cells kept at 28:28 ºC reveals that at a constant temperature of 28ºC, nighttime biomass loss was more affected by temperature than by daily yield. On the other hand, the low dispersion of the data points of the cells kept at low temperature, reveals that cells kept constantly at 18:18 ºC had their yield and nighttime biomass loss inhibited. These observations suggest that temperature can influence nighttime biomass loss as well as productivity, even though these factors (nighttime biomass loss and daylight productivity) seem to be correlated.

A summary of the daily areal productivities and the corresponding nocturnal biomass loss is shown in Supplementary Table S2. Although the light period in both trials was equally controlled (from 8 am to 6 pm), due to differences in the amount of light energy and intensities that occurred between the two trials, gross areal productivities (g m^{-2} day^{-1}) of both cultures were normalized to their corresponding daily energy received (MJ m^{-2} day^{-1}). Cultures grown according to the temperature regime of 28:18 ºC L:D (control) showed comparable biomass growth yields between the first (1.517 g MJ^{-1}) and second trial (1.475 g MJ^{-1}), indicating that the light utilization efficiency of the cultures was very similar (Supplementary Table S2). However, the culture grown at 18:18 ºC, L:D presented a much lower value (0.987 g MJ^{-1}) indicating a clear effect of the low temperature on daylight growth (Supplementary Table S2).

Daylight biomass productivity showed a good correlation to light irradiance. Indeed, higher daylight productivities were recorded with cultures grown under higher light irradiance that occurred during the first trial (July 26th -August 3rd, 2018), with a mean value of 30.8 g m^{-2} day^{-1} for the culture maintained at the dual temperature (28:18 ºC L:D), and 27.8 g m^{-2} day^{-1} for the one maintained at the constant temperature of 28 ºC (28:28 ºC L:D). However, due to the impact of night biomass loss, the net productivity was reduced to 14.5 and 16.6 g m^{-2} day^{-1} respectively (Supplementary Table S2). In September, although the light irradiance declined significantly, the net productivity of the culture maintained at 28:18 °C L:D was slightly better (16.1 g m^{-2} day^{-1} in September vs 14.5 g m^{-2} day^{-1} in August). This may indicate that the growth of *Nannochloropsis* gets saturated over about 16 MJ m^{-2} day^{-1}. This finding is supported by previous research carried out by Zittelli et al. (1999) who found, in a two-year experiment, better yields in *Nannochloropsis* in September, when light irradiance declined to

about 13 MJ m^{-2} day^{-1} [28]. A close relationship between light intensity (at which cultures were exposed) and dark respiration was reported elsewhere [33,34]. Night biomass loss was mostly related to daylight temperature rather than to nocturnal temperature. Biomass loss in *N. oceanica* was always very impressive ranging between 19% and 53% of the biomass synthesized during the daylight period (Supplementary Table S2).

3.2. Fluorescence parameters

The maximum quantum efficiency of PSII (F_v/F_m) showed a steady trend with higher values in the morning and lower in the evening, for all experiments (see Table 2), thus indicating that photosynthetic activity was probably downregulated in the middle of day due to the prevailing high light [35]. This trend for the maximum photochemical efficiency was previously observed in *Nannochloropsis* sp. grown in two different PBRs outdoors [36].

Table 2 Daily average (± SD) of maximum quantum efficiency of PSII (F_v/F_m) values of *Nannochloropsis oceanica* cells grown under different temperature regimes in outdoors tubular photobioreactors in semi-continuous cultivation mode. Statistical differences between the 28:28 ºC L:D and the control of Trial 1, and the 18:18 ºC L:D and the control of Trial 2 are depicted with "*" (*n*=8).Values of F_v/F_m are significantly different (p<0.05) from morning to the corresponding evening for all conditions.

Time of day	**Trial 1**		**Trial 2**	
	28:18 ºC L:D	28:28 ºC L:D	28:18 ºC L:D	18:18 ºC L:D
Morning	0.680 ± 0.024	0.672 ± 0.018	0.679 ± 0.021	0.630 ± 0.016 *
Evening	0.633 ± 0.031	0.621 ± 0.032	0.634 ± 0.045	0.570 ± 0.023 *

Cultures maintained at the constant temperature of 18 ºC (overnight and at daytime) caused a negative impact upon the PSII performance, compared to the others. Lower daily temperature can promote sensitivity to light stress – and accordingly slow down the photosynthetic rate, thus inhibiting PSII repair mechanisms, and hampering the protective mechanisms underlying photoinhibition prevention [35]. This higher susceptibility to photoinhibition when the culture was maintained at lower temperatures can explain the lower productivity of microalgal cells under this condition. Similar results of lower F_v/F_m were found for *A. platensis*, when kept at suboptimal temperatures [35].

Fast fluorescence curves of the 2nd and 7th day of each trial are depicted in Figure 4.

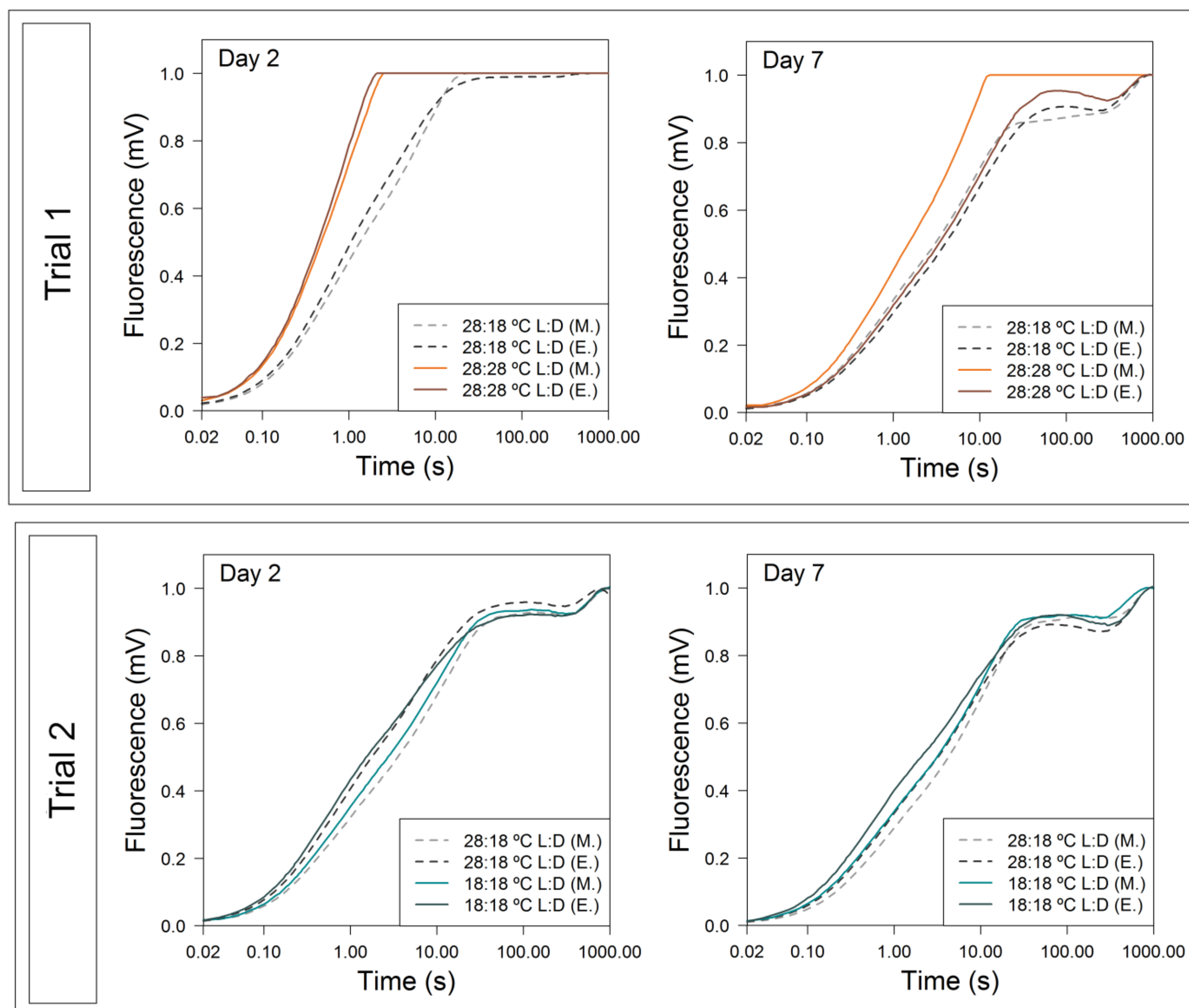

Figure 4 Kinetic representation of the OJIP transients from *Nannochloropsis oceanica* grown in tubular photobioreactors outdoors under semi-continuous regime and subjected to different temperatures (*n*=3). The first row shows the OJIP curves for the morning (M.; orange) and evening (E.; brown) samples of the 2nd and 7th days of the cells kept at 28:28 ºC L:D (solid lines) in Trial 1. The second row shows the results of the second trial when cells were kept at 18:18 ºC L:D (solid lines) in the morning (M.; light blue) and evening (E.; dark blue). Control curves (28:18 ºC L:D) are represented as dashed lines for morning (M.; light grey) and evening (E.; dark grey) samples in both trials. Curves were double normalized to F_0 and F_m, when possible.

The culture maintained at 28:28 °C L:D revealed a very high slope of the O-J fluorescence rise relative to the control, thus making it impossible to normalize fluorescence intensity of the P point in the OJIP transient for this culture (Figure 4, Trial 1). This effect can also be detected in Figure 5 (where the OJIP parameters are represented as ratios to the corresponding controls), where the values of V_j and M_0 are more than 1.5-fold those of the control. M_0 represents net rate of the closure of PSII RCs [37],

which was fastest in the cells grown at 28:28 ºC L:D, particularly after the dark period. Given that 1 – $V_J = ET_0/TR_0$ (also referred to as ψ_{E0}), one concludes that the transfer efficiency of a PSII-trapped electron from Q_A^- to plastoquinone (PQ) is lowest in the beginning of the run in the first trial. Nonetheless, microalgae kept at 28:28 ºC L:D overcame the damage caused by the high temperature stress during the dark period by day 7. A relation between high temperature damage to PSII and V_j was previously reported in *Nannochloropsis* sp. [36]. The F_v/F_0 values are proportional to the activity of the water-splitting complex on the donor side of PSII, and were suppressed at the beginning of the first trial due to the warmer temperatures overnight (28:28 ºC L:D) – but also approached the control values by the end of the experiment.

Concerning the energy fluxes per absorbed photon flux, the maximum quantum yield of primary photochemistry that leads to reduction of pheophytin and Q_A ($\Phi_{P0} = TR_0/ABS = F_v/F_m$) was supressed in the beginning for the cells kept at 28:28 ºC L:D, but came close to control values only in the end. The same pattern was found for the quantum yield of electron transport ($\Phi_{E0} = ET_0/ABS$), where the probability that an absorbed photon will move an electron into the electron transport chain was lowest in the beginning but eventually recovered, reaching values closer to the control. Reaction centre densities (RC/CS_m) were a bit lower than the control, yet they remained constant along the trial.

Phenomenological energy fluxes per excited cross section, at completely closed RC, were also affected by high temperatures in the beginning when the culture was kept at 28:28 ºC L:D. Even though absorption by the PSII antenna pigments (ABS/CS_m) was unaltered, the trapped photon flux used for the primary charge separation and stabilization of the RCII for closing all PSII RCs (TR_o/CS_m) decreased in the beginning, when cells were kept at 28:28 ºC L:D. However, the overall electron transport flux (ET_o/CS_m) was strongly affected right from the start, until the end of the experiment, in the case of cells kept at higher temperatures for being unable to perform as well as microalgae grown under control conditions. As significant changes in electron transport flux are mirrored by the variation of the fraction of reduced Q_A [37], this would explain the fast rise of the transients at the beginning of the first trial. Excitation energy dissipation (DI_0/CS_m) was higher in the beginning of the trial. Apart from

the morning samples after the culture was kept at 28 ºC overnight, the values for the dissipation flux reverted more closely to the control values.

Finally, the performance index on absorption basis (PI_{ABS}; [38,39]) reflects the functionality of both PSII and PSI, thus providing information on the current state of algae performance under stress conditions [40]. Compared to the control, the cells kept at 28:28 ºC L:D temperatures revealed high stress from the beginning, recovering towards the end. Nonetheless, the cells kept under constant 28 ºC were apparently unable to deal with the high temperature of the dark periods; hence, PI_{ABS} was more sensitive than Φ_{P0} (F_v/F_m).

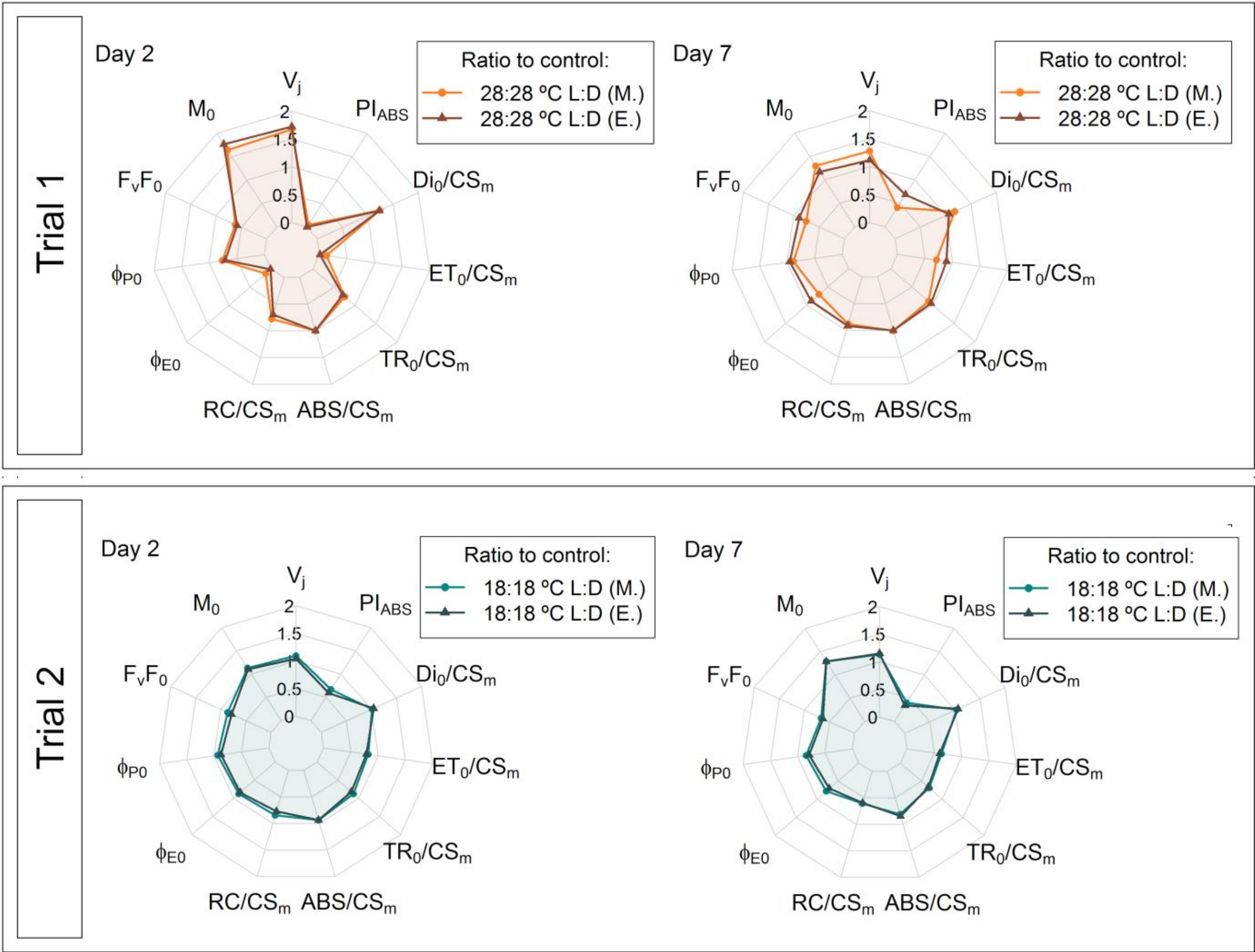

Figure 5 Temperature influence on OJIP determined parameters of *Nannochloropsis oceanica*, grown in tubular photobioreactors outdoors under semi-continuous regime and subjected to different temperatures (n = 3). Spider plots depict the ratio of the conditions (28:28 ºC L:D in the first trial, and 18:18 ºC L:D in the second trial) to their corresponding controls (28:18 ºC L:D). The first row shows the results of Trial 1 on the 2nd and 7th day, respectively, with morning (M.; orange) and

evening (E.; brown) samples, and of Trial 2 on the second row, also with morning (M.; light blue) and evening (E.; dark blue) samples for each day.

Although slightly lower, ratios to the control in the beginning of the second trial were quite similar with the exception of F_v/F_0, RC/CS_m, ET_0/CS_m and PI_{ABS} (Figure 5, Trial 2). At the end of this trial, all ratios to the control dropped, except for M_0 and V_j that increased. This limitation within electron transport could be explained by a diminished RC density, caused by the cold temperature the cells were exposed to (18:18 ºC L:D). This could reflect a self-defence mechanism to avoid low-temperature induced photoinhibition, already reported in PAM measurement of F_v/F_m [41].

3.3. Photosynthetic and respiration measurements

The relation found between respiration and net photosynthesis lies within the expected values [8], but the correlation between nighttime biomass loss and respiration values by the end of the dark period (morning) found using all data points revealed a high dispersion ($R(27)=0.64$); nevertheless, the correlation was found to be significant ($p<0.05$; plot in supplementary Figure S1). Overall, respiration values in the first trial started to increase with time for both cultures (Figure 6). However, oxygen production doubled after the evening of day 2 and remained stable until the morning of the 7th day. Gross O_2 production (O_2 production plus respiration rate) was mostly higher for the culture kept at 28:18 ºC L:D (control). On the other hand, the respiration values increased from the beginning to the end of the trial under both conditions.

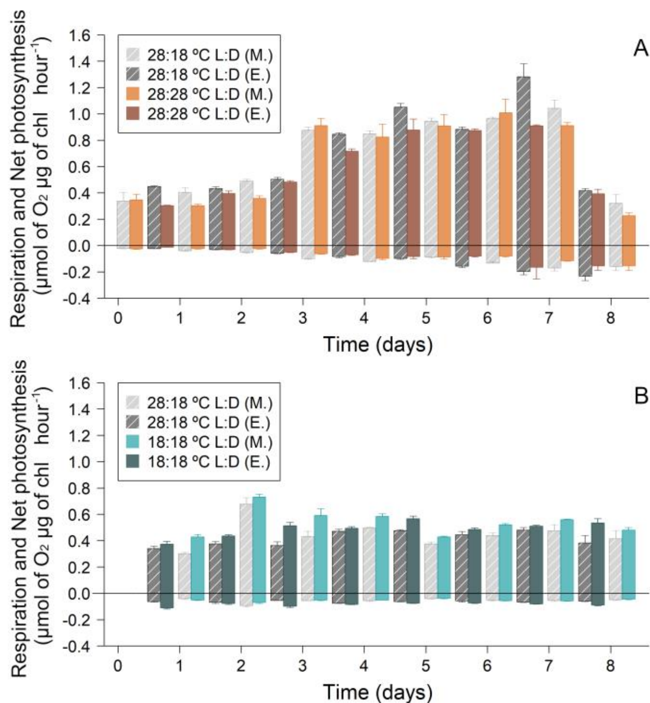

Figure 6 Net photosynthetic oxygen evolution (y>0) and respiration (y<0), normalized to chlorophyll of *Nannochloropsis oceanica* grown in semi-continuous mode in outdoor tubular photobioreactors at different temperatures, showing Trial 1 (A) and Trial 2 (B). Values for cells grown at 28:28 ºC L:D or at 18:18 ºC L:D are depicted as orange (lighter for morning [M.] and darker for evening [E.] samples) or blue (lighter for morning [M.] and darker for evening [E.] samples) bars, respectively. Grey stripped shaded bars correspond to the values of the corresponding controls, kept at 28:18 ºC L:D(n=3).

In the culture maintained at 28:28 °C (L:D), oxygen production and consumption rates were more stable along the experiment. Net oxygen production was higher for cells cultivated at 18:18 ºC (L:D) when compared to the control (28:18 °C, L:D). Respiration values in the morning were very similar under both conditions; however, the cells kept at 18 ºC after the light period showed a slightly higher respiration rate. This apparent contradiction is explained by the fact that cells grown at 18 °C, were characterised by a 20% higher spectral averaged optical cross-section of chlorophyll *a* compared to the 28:18 °C L:D (results not shown), and by a lower cellular chloropyll content (see section 3.4.). Moreover, the oxygen measurements were carried out at 28 ºC which may have allowed the cell to recover its photosynthetic capacity. A previous study using *N. oculata* reported a different behaviour at the beginning of the light period, with the increase of O_2 production levels being slower when the cells were kept at 25:15 ºC (L:D periods) than at 20:20 ºC (L:D periods), due to a reduced net photosynthesis [31]. In our study, the low temperature of 18 ºC maintained in the culture at 18:18 °C

(L:D) did not seem to limit electron transport or ability of light use by the microalga when transferred to 28 °C, thus indicating that photosynthetic activity can recover quickly.

3.4. Chlorophyll content

The cell chlorophyll content was tendentially higher after the light period for both trials, as expected, owing to the high irradiation to which they were subjected. This is consistent with previous reports on *Nannochloropsis* sp., subjected to a photoperiod containing L:D cycles (Figure 7) [19,20]. However, if chlorophyll content was expressed per biomass DW it would be highest at the end of the dark period, since biomass DW dropped during the night.

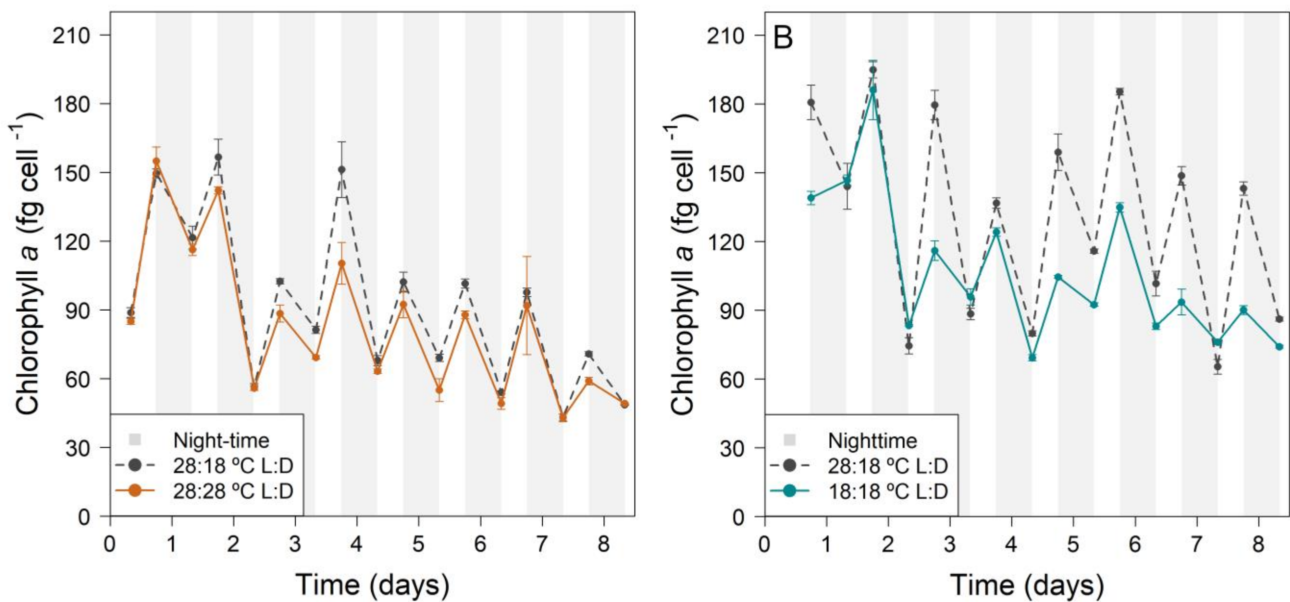

Figure 7 Chlorophyll *a* content per cell of *Nannochloropsis oceanica*, grown in outdoor tubular photobioreactors under semi-continuous regime and subjected to different temperatures, showing Trial 1 (A) and Trial 2 (B). Grey lines represent the controls when cells were kept at 28:18 ºC L:D, and orange and blue lines represent the cells kept at 28:28 ºC L:D and 18:18 ºC L:D for each trial, respectively. Grey bars represent the dark period, when the cultures were covered (n=2).

In the culture maintained at constant temperature of 28 °C along the diel cycle, a slightly lower chlorophyll production per cell was observed compared to the control (28:18 ºC L:D). Nonetheless, chlorophyll *a* production was noticeably more suppressed when the cells were grown at 18:18 ºC L:D in the second trial. This finding was expected, as low temperature-acclimated algae tend to have lower contents of photosynthetic pigments; and higher temperature-grown cultures usually exhibit higher

chlorophyll production [41–43]. Lower temperatures can, in general, inhibit chlorophyll build-up by decreasing the light harvesting complex, thus affecting the values of maximum photochemical efficiency of PSII in the cells kept at 18:18 ºC L:D, see discussion above [44].

3.5. Lipids

The lipid content per cell of *N. oceanica* exhibited a trend similar to that of chlorophyll *a*, being highest by the end of the light period, in both runs and for both conditions (Figure 8).

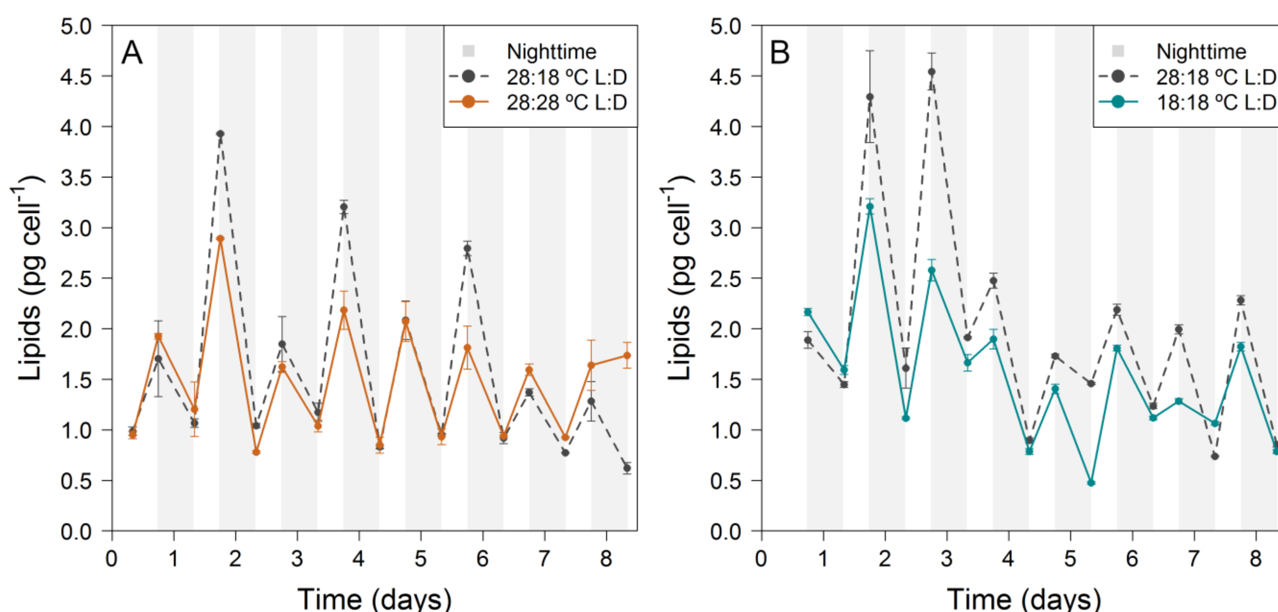

Figure 8 Temperature influence on the lipid content of *Nannochloropsis oceanica* cells, grown in tubular photobioreactors outdoors under semi-continuous regime and different temperature profiles(*n*=3). In Trial 1 (A) the culture was kept at 28:28 ºC L:D (orange line), and in Trial 2 (B) the cells were kept at 18:18 ºC L:D (blue line). Grey lines represent the controls (28:18 ºC L:D) for each trial, and light grey bars represent the dark *period.*

This finding supports the idea that cells consume a part of their lipid reserves to support the energy demand associated to cell division taking place at night. This observation has been previously reported in *Nannochloropsis* sp. synchronized cultures, where lipids are the main energy reserve [19,20]. A similar pattern was also found in outdoor cultures of *Phaeodactylum tricornutum* [45]. The lipid content was higher in the cultures kept at dual temperatures (28:18 °C, i.e., control), in both trials; while a slower lipid synthesis was observed for the cells kept at 18 ºC. Previous experiments showed a higher

lipid production by *N. oculata* cultures, when temperature was increased from 20 to 25 ºC [46]. In addition, a strong correlation was found between higher temperatures and lipid contents in *N. salina* [47]. The lipid content in *Nannochloropsis* sp. usually increases under stress conditions, mostly nutrient limitation, yet this does not hold for low temperature-stress (18 °C).

3.6. Fatty acids

The FAME profile of *N. oceanica* was mainly composed of myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1) and eicosapentaenoic (C20:5n-3, EPA) acids, while linoleic (C18:2n-6), oleic (C18:1), stearic (C18:0) and arachidonic (C20:4n-6, AA) acids were detected to a lower extent (Table 3). Because of the high myristic and palmitic acids contents, saturated fatty acids (SFA) represented around half of the total FAME detected in all growth conditions tested. The FAME profile of cultures, from the beginning until the end of each trial, was relatively stable under all conditions, due to the stable culture conditions (light, temperature, dilution rate). Overall, the FAME profile was similar to those previously reported for *Nannochloropsis* species [9,48].

Table 3 Daily average (± SD) of fatty acid composition as percentage of total fatty acids of *Nannochloropsis oceanica*, grown in a semi-continuous cultivation mode in tubular photobioreactors outdoors under different temperature regimes. For each trial, statistical differences between the 28:18 ºC L:D or 18:18 ºC L:D regimes and their corresponding controls (28:18 ºC L:D) are marked with an "*" (p<0.05; trial 1 n=8; trial 2 n=7).

Fatty Acid (%)	**Trial 1**				**Trial 2**			
	28:18 ºC L:D		**28:28 ºC L:D**		**28:18 ºC L:D**		**18:18 ºC L:D**	
	Morning	Evening	Morning	Evening	Morning	Evening	Morning	Evening
C14:0	7.49 ± 0.52	7.36 ± 1.05	7.48 ± 0.65	6.59 ± 0.60	7.47 ± 0.81	7.88 ± 0.98	5.97 ± 0.63 *	6.33 ± 0.89 *
C16:1	18.87 ± 0.97	16.53 ± 0.56	18.37 ± 0.65	16.28 ± 0.82	17.88 ± 1.48	17.02 ± 1.52	19.51 ± 0.86	20.49 ± 1.45 *
C16:0	43.67 ± 4.28	53.41 ± 4.19	45.12 ± 6.23	52.75 ± 5.02	46.22 ± 6.63	52.57 ± 2.31	37.75 ± 6.35	43.89 ± 4.61 *
C18:2n-6t	2.75 ± 0.39	1.44 ± 0.41	2.43 ± 0.35	1.78 ± 0.36	2.06 ± 1.04	1.76 ± 0.55	2.26 ± 0.78	1.56 ± 0.47
C18:1c	3.06 ± 0.44	2.35 ± 0.16	3.07 ± 0.16	2.37 ± 0.13	2.61 ± 0.58	2.40 ± 0.24	2.03 ± 0.28 *	1.97 ± 0.19 *
C18:0	1.00 ± 0.16	3.37 ± 0.59	1.21 ± 0.27	3.00 ± 0.51	1.11 ± 0.40	2.88 ± 0.71	0.85 ± 0.49	1.44 ± 0.58 *

C20:4n-6	3.74 ± 1.17	1.87 ± 1.06	3.62 ± 1.67	2.76 ± 1.69	2.52 ± 1.35	1.76 ± 0.68	3.80 ± 1.17	3.05 ± 1.23 *
C20:5n-3	19.42 ± 3.44	14.34 ± 2.26	18.7 ± 5.27	14.46 ± 3.45	18.33 ± 1.73	13.90 ± 2.07	26.99 ± 3.41 *	20.05 ± 2.77 *

Lower growth temperatures led to a significant decrease of myristic, palmitic, oleic and stearic acids, and a consequent increase of AA, EPA and palmitoleic acid content. The increase of polyunsaturated fatty acids (PUFA), at the expense of SFA, under lower temperature conditions is in agreement with previous reports [9,28,44,49]. Interesting enough, is the higher content of EPA observed in the culture maintained at the suboptimal temperature of 18 ºC by day and by night, which reached 20% of the total fatty acids at the end of the light period and rose up to 27% in the following morning. This difference can contribute greatly to increase the value of the biomass particularly when harvested in the morning. Nonetheless, attention must be paid to the balance between biomass productivity and EPA content of the biomass, in order to obtain the highest EPA productivity possible.

Analysis of the daily-averaged fatty acid changes showed a sizeable decrease in EPA and total PUFA percentage in the evening, under all conditions; while palmitic acid and SFA were, in general, more represented in the evening (Figure 9). On the other hand, monounsaturated fatty acids (MUFA) did not show major changes between morning and evening samples. The decrease of EPA content with increasing radiation, and consequent accumulation in the dark phase have been also reported elsewhere for *Nannochloropsis* sp. [9,20].

The observed diel changes in FA levels can be explained by *Nannochloropsis* cells accumulating storage lipids (SFA) during daytime, which are later metabolized during nighttime to generate the necessary ATP levels for the cell to survive during the dark period, synthesize DNA, and enter mitosis [50]. Conversely, EPA increases during the dark period, when cell division occurs, since PUFA are important constituents of the thylakoid membranes [9,20]. Regarding the effect of temperature over the diel cycle, no differences were found in the cells maintained at 28 ºC throughout the day and night periods, and those subjected to 28:28 ºC (L:D). However, cultures permanently kept at 18 ºC showed

lower SFA, and increased contents of PUFA and MUFA in the evening, perhaps a metabolic response that counteracts a decrease in membrane fluidity in cells under this environmental condition.

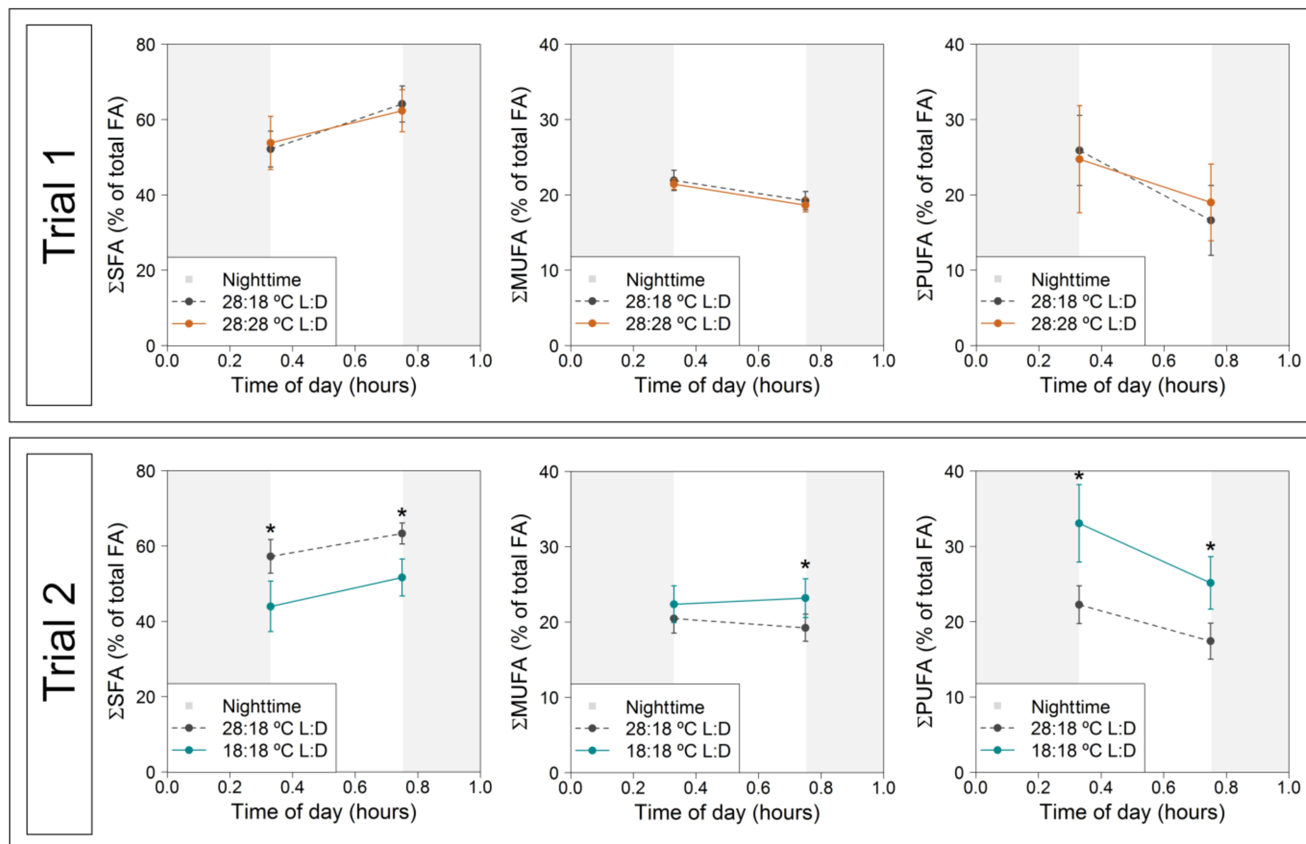

Figure 9 Daily average (± SD) of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) for morning and afternoon samples of *Nannochloropsis oceanica*, grown in a semi-continuous cultivation mode in tubular photobioreactors outdoors under different temperature regimes. The first row shows Trial 1, with the orange solid lines representing the cells kept at 28:28 ºC L:D. The second row shows Trial 2, with the blue solid lines representing the cells kept at 18:18 ºC L:D. For both trials, dashed grey lines represent the corresponding controls (28:18 ºC L:D). Statistical differences between the tested condition and the respective control are marked with an "*" ($p<0.05$; trial 1 n=8; trial 2 n=7).

The ratio of unsaturated fatty acids to SFA was also highest when the culture was kept at 18 ºC constantly, but with low values of 1.24 and 0.94 in the morning and afternoon, respectively. A previous report also claimed a minor influence of temperature upon this ratio in *N. salina* [25].

4. Conclusions

Unlike one might expect, higher night biomass losses were observed especially for the cultures operated at dual temperatures of 28 °C during daytime, and 18 °C during nighttime. Suboptimal

temperature (18 °C during all the diel cycle) led to lower biomass productivity, but also lower biomass loss overnight. The results gathered in this work support a strong correlation between daily yield and nighttime biomass loss, in cultures operated in a semi-continuous regime. However, this relation can be influenced by temperature. Relative EPA content was always higher in the morning cultures suggesting that, in accordance with productivity, harvesting should be performed preferably in the early morning hours. Although the cells kept constantly at 28 ºC experienced some stress as revealed by F_v/F_m reduction, fluorescence measurements revealed that they fully recovered overnight. On the other hand, those kept at 18 ºC throughout the day and night periods underwent a decrease in F_v/F_m, coupled to a lower photosynthetic activity and biomass productivity. Nonetheless, *N. oceanica* tolerated a wide range of temperatures – which is a desirable trait for biomass production.

Chlorophyll production was highest at the end of daily radiation exposure, and overall lowest in the culture grown at 18 ºC. Lipids synthesized during the day were partially consumed overnight to support cell division. PUFA production reached a maximum after the dark period, as opposed to SFAs. This effect is most likely a consequence of plastidial biogenesis, which is tightly linked to cell division. Lower temperatures led to higher EPA accumulation, which represented most of the PUFA profile, a trait that increases the economic value to the biomass.

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Declaration of contributions

MC, BC, GCZ, JV FXM and GT have worked in the conception and design of the experiments. MC and BC performed the experiments. MC, BC, IM, HP, GCZ and GT contributed to the collection, assembly and interpretation of data. MC, BC, IM, HP, JV and GT aided in the drafting of the manuscript and GCZ and FXM performed critical revisions thereof. Funding for the experiment and analysis was supported by GCZ, JV, FXM and GT through project grants under their coordination. All authors approved the final version of the article.

Conflict of interest statement

We declare that this manuscript has not any potential financial or other interests that could be perceived to influence the outcomes of the research.

Statement of informed consent, human/animal rights:

No conflicts, informed consent, human or animal rights applicable.

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Supplementary data

Title: Effect of temperature on growth, photosynthesis and biochemical composition of *Nannochloropsis oceanica*, grown outdoors in photobioreactors

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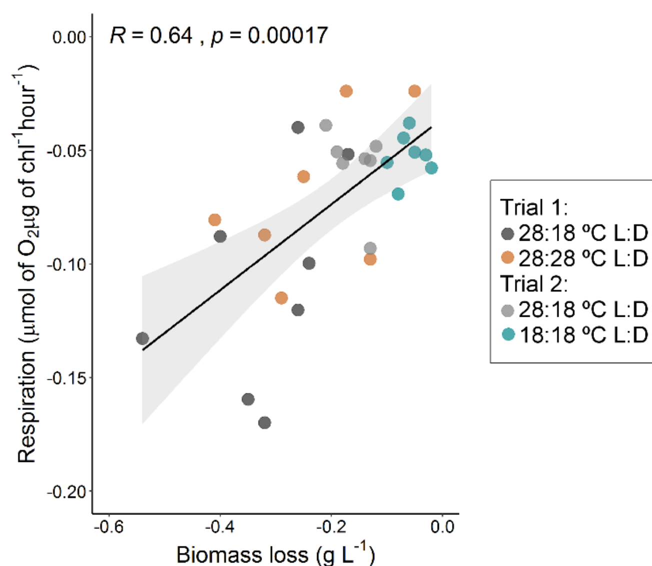
Supplementary Table S1 Temperature influence on daily volumetric productivity and nighttime biomass loss (± SD) in *Nannochloropsis oceanica*, grown outdoors in a semi-continuous mode, under different temperature regimes in two independent trials. Daily volumetric productivity (g L^{-1} d^{-1}) was determined by subtracting the final dry biomass concentration of the evening (g L^{-1}) with the dry biomass concentration in the morning of the same day (after dilution; g L^{-1}) and dividing by the determined period (in this case, one day). Daily volumetric nighttime biomass loss (g L^{-1} d^{-1}) was determined similarly, but by subtracting the final dry biomass concentration of the evening (g L^{-1}) with the dry biomass concentrations of the following morning (before dilution; g L^{-1}) and dividing by the same period. Nighttime biomass loss (%) was determined by subtracting the final dry biomass concentration of the evening (g L^{-1}) with the dry biomass concentrations of the following morning (before dilution; g L^{-1}) and dividing by the same value of final dry biomass concentration in the evening (g L^{-1}).

Trial 1						
Time (days)	**Daily volumetric productivity (g L^{-1} d^{-1})**		**Daily volumetric nighttime biomass loss (g L^{-1} d^{-1})**		**Nighttime biomass loss (%)**	
	28:18 °C (L:D)	28:28 °C (L:D)	28:18 °C (L:D)	28:28 °C (L:D)	28:18 °C (L:D)	28:28 °C (L:D)
0	0.496 ± 0.111	0.596 ± 0.061	0.260 ± 0.125	0.173 ± 0.105	27.7 ± 13.7	16.1 ± 9.79
1	0.436 ± 0.071	0.484 ± 0.028	0.170 ± 0.091	0.050 ± 0.076	21.0 ± 11.3	5.21 ± 7.93
2	0.615 ± 0.014	0.539 ± 0.028	0.240 ± 0.045	0.250 ± 0.051	22.9 ± 4.27	23.6 ± 4.85
3	0.706 ± 0.071	0.570 ± 0.042	0.260 ± 0.072	0.130 ± 0.042	23.0 ± 6.54	13.1 ± 4.32
4	0.657 ± 0.057	0.532 ± 0.014	0.400 ± 0.102	0.320 ± 0.045	37.0 ± 9.64	31.7 ± 4.45
5	0.784 ± 0.042	0.654 ± 0.099	0.540 ± 0.045	0.410 ± 0.099	47.8 ± 4.35	39.8 ± 10.3
6	0.553 ± 0.028	0.569 ± 0.028	0.320 ± 0.040	0.290 ± 0.032	38.1 ± 4.93	32.2 ± 3.66
7	0.540 ± 0.028	0.367 ± 0.000	0.350 ± 0.032	0.115 ± 0.007	39.8 ± 3.81	15.5 ± 0.956
Daily average	*0.598 ± 0.107*	*0.539 ± 0.080*	*0.318 ± 0.107*	*0.217 ± 0.113*	***32.2 ± 9.20***	***22.2 ± 10.9***
Trial 2						
Time (days)	**Daily volumetric productivity (g L^{-1} d^{-1})**		**Daily volumetric nighttime biomass loss (g L^{-1} d^{-1})**		**Nighttime biomass loss (%)**	
	28:18 °C (L:D)	18:18 °C (L:D)	28:18 °C (L:D)	18:18 °C (L:D)	28:18 °C (L:D)	18:18 °C (L:D)
1	0.426 ± 0.042	0.255 ± 0.057	0.130 ± 0.121	0.080 ± 0.080	14.3 ± 13.3	10.3 ± 10.3
2	0.459 ± 0.042	0.415 ± 0.000	0.130 ± 0.051	0.030 ± 0.014	14.0 ± 5.52	3.41 ± 1.61
3	0.527 ± 0.085	0.373 ± 0.014	0.180 ± 0.089	0.050 ± 0.032	18.0 ± 9.07	5.49 ± 3.48
4	0.449 ± 0.085	0.238 ± 0.042	0.210 ± 0.086	0.060 ± 0.045	23.3 ± 9.81	7.79 ± 5.82
5	0.468 ± 0.042	0.330 ± 0.014	0.190 ± 0.051	0.100 ± 0.045	21.3 ± 5.82	12.0 ± 5.39
6	0.507 ± 0.028	0.336 ± 0.028	0.140 ± 0.028	0.020 ± 0.117	14.9 ± 3.04	2.50 ± 14.6
7	0.452 ± 0.028	0.254 ± 0.071	0.120 ± 0.040	0.070 ± 0.076	13.0 ± 4.37	8.86 ± 9.67
Daily average	*0.470 ± 0.033*	*0.314 ± 0.062*	*0.157 ± 0.033*	*0.059 ± 0.026*	***17.0 ± 3.71***	***7.19 ± 3.28***

Supplementary Table S2 Averaged daily areal productivities (gross and net) and nighttime biomass loss (± SD) of *Nannochloropsis oceanica* cultures grown under different temperature regimes in tubular photobioreactors (PBR) outdoors in two independent trials. Gross areal productivity (g m^{-2} d^{-1}) was determined by dividing the daily average biomass produced (g d^{-1}) with the PBR's area (m^2). Growth yield (g MJ^{-1}) was obtained by dividing the gross areal productivity with the daily irradiance. Net areal productivity (g m^{-2} d^{-1}) was determined by subtracting the gross areal productivity with the night biomass loss (g m^{-2} d^{-1}). The nighttime biomass loss was calculated by dividing the daily average biomass loss (g d^{-1}) with the PBR's area (m^2); in brackets is reported the percentage of biomass loss regarding the biomass synthesized during the light period.

Trial	Condition	Mean daily irradiance (MJ m^{-2} day^{-1})	Gross areal productivity (g m^{-2} d^{-1})	Growth yield (g MJ^{-1})	Net areal productivity (g m^{-2} d^{-1})	Nighttime biomass loss (g m^{-2} d^{-1})
1	28:18 °C L:D	20.3 ± 3.21	30.8 ± 5.53	1.52 ± 0.36	14.5 ± 7.81	16.4 ± 5.52 (53.1%)
	28:28 °C L:D		27.8 ± 4.11	1.37 ± 0.30	16.6 ± 7.13	11.2 ± 5.82 (40.3%)
2	28:18 °C L:D	16.4 ± 4.03	24.2 ± 1.68	1.48 ± 0.38	16.1 ± 2.39	8.10 ± 1.69 (33.5%)
	18:18 °C L:D		16.2 ± 3.21	0.99 ± 0.31	13.2 ± 3.48	3.02 ± 1.33 (18.6%)



Supplementary Figure S1 Correlation between biomass loss (g L^{-1}) and respiration values (µmol of O_2 µg of chlorophyll^{-1} hour^{-1}), combining data points from both trials and conditions using *Nannochloropsis oceanica*, grown outdoors in tubular photobioreactors under different temperatures. Data points from the 28:18 ºC L:D (controls) kept cultures are depicted in dark (Trial 1) and light (Trial 2) grey; cells kept at 28:28 ºC L:D (Trial 1) and 18:18 ºC L:D (Trial 2) are depicted in orange and blue. respectively. The shaded grey area represents the confidence interval (95%) of Pearson's correlation. The correlation coefficient (R = 0.64) and the significance level ($p<0.05$) are shown in the upper left corner of the plot (n=29).