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

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Abstract

Temperature is an important factor affecting growth, photosynthetic rate and biomass composition. As such, this study focused on the effects of temperature on biomass yield and night-time biomass loss, as well as photochemical changes, using *Nannochloropsis oceanica* as model species, grown in two outdoor 50-L tubular photobioreactors (PBR). Microalgal biomass produced during daytime is partially lost overnight by respiration of its intracellular carbon reserves, which may have a considerable negative impact on industrial biomass productivity. 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 the early morning and evening. Chlorophyll fluorescence quenching analysis was also used to better evaluate their physiological state. Our results revealed that *N. oceanica* shows a wide temperature tolerance with relevant night-time biomass loss, which decreased at lower temperatures, although at the expenses of 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. Total lipids were 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 industrial scale development.

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 indeed bring about a decrease in biomass productivity during the day, as well as an increased night-time biomass loss [3,4]. In fact, cells metabolize a wide array of reserve biomolecules at night, in order to produce maintenance energy and support cell division, thus leading to biomass loss [5]. Previous reports have described night-time 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 night-time 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 assess the influence of suboptimal temperature on growth, night-time biomass loss and biochemical composition 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 night-time (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)	Night-time temperature (6 pm-8 am)	Experimental period	Mean daily light 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), Tris-HCl (20 mM) and 10 times more the values of $NaNO_3$ (17.7 mM) and NaH_2PO_4 (0.84 mM) when compared to 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, every day, a part of the culture was removed 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), which is 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 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 dried afterwards in the oven at 105 ºC until constant weight. Cell counts were carried out in duplicate, using a Bürker counting chamber. Night-time 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 by using 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 measuring light (0.5 µmol m^{-2} s^{-1}) was applied to measure (F_0). A strong saturating pulse (6000 µmol m^{-2} s^{-1}) was then applied 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 [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 production 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) light 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 rate 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 of chlorophyll^{-1} 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):

$$\text{Chlorophyll } a \text{ (µg mL}^{-1}) = 11.64 \, (A_{663} - A_{750}) \; ; \qquad (1)$$

the equation was shortened, 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 according to a modified protocol, based on Folch (1957), Lepage and Roy (1984), 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 mix 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 (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 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, were 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 night-time 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].

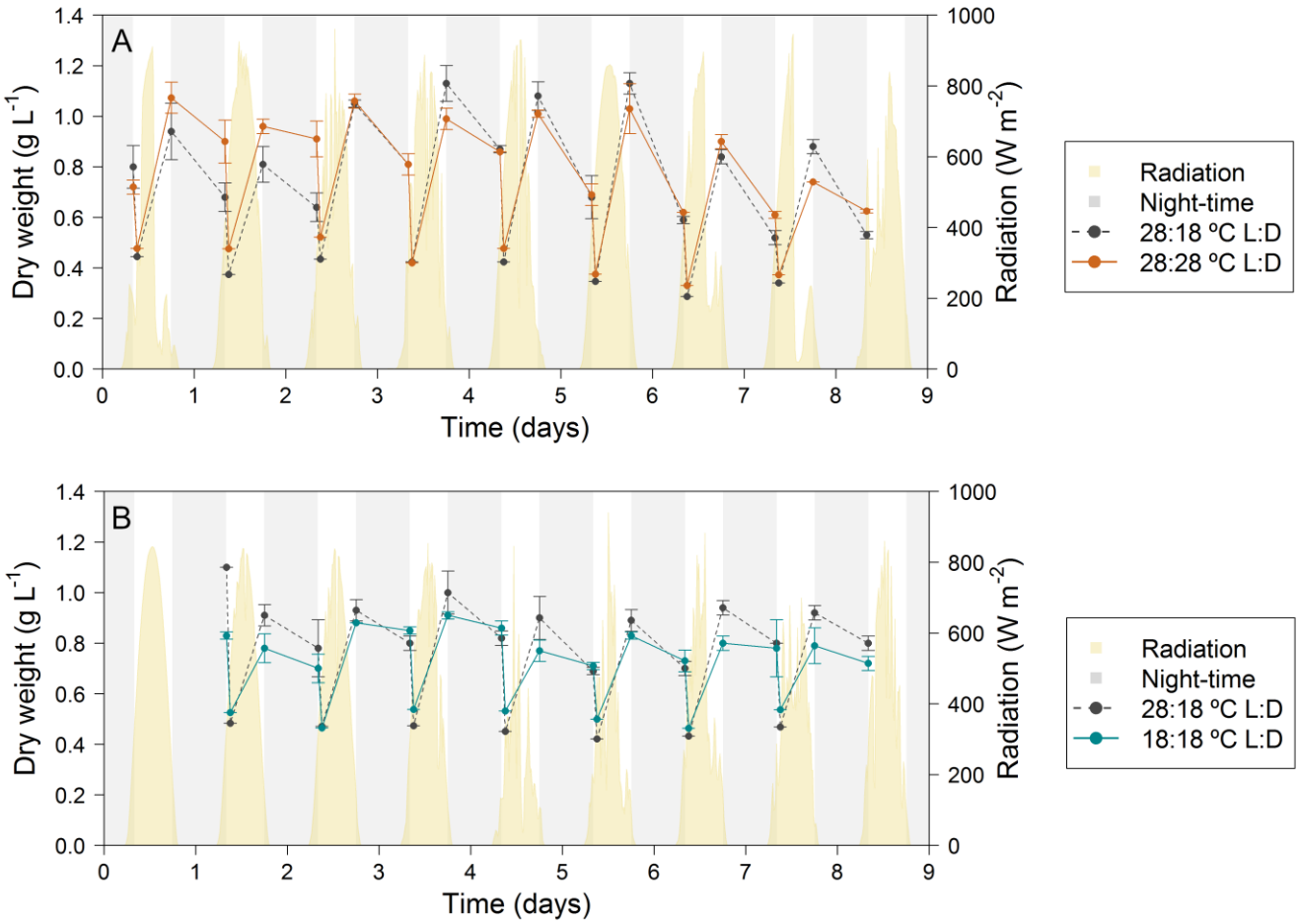

Figure 1 Changes in *Nannochloropsis oceanica* biomass dry weight (DW) grown outdoors in a semi-continuous mode under different temperature regimes. After the morning sample, cultures were diluted to 0.45 g L^{-1} of DW and allowed to grow until the following morning. Both the first (A) and second (B) 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 measured values of DW 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 clearer view of biomass variations for each temperature condition each day was establish 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 night-time biomass losses, reaching up to 53% and 33% of the daily produced biomass for trial 1 and

2, respectively. Differences between controls are ~~probably~~ a result of a slightly higher solar irradiation in the first trial when compared to the trial 2 – the cultural period of which (August to September 2018) was characterised by unstable weather conditions. Detailed information on daily productivities and night losses can be found in the Supplementary Table S1.

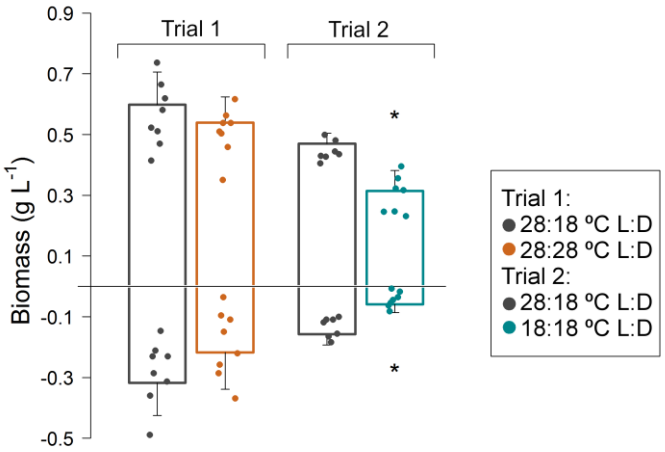

Figure 2 Daily average (+SD) of biomass dry weight (DW) changes in *Nannochloropsis oceanica* grown outdoors in a semi-continuous mode, under different temperature regimes, in two sequential trials (trial 1 n=8; trial 2 n=7). Positive and negative values represent the daily biomass yield and loss, respectively. 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 night-time loss. However, in other studies this difference was more pronounced, where using *N. salina*, higher biomass losses at higher temperatures in the dark were found [7], as well as in previous experiments with *Chlorella pyrenoidosa*, *Phaeodactylum tricornutum* and *A. platensis,* in which lower night temperatures led to lower biomass loss [4,5,21].

The slowest growth was achieved by the cultures maintained at 18:18 ºC L:D which can be considered a low temperature for *Nannochloropsis* spp. growth, known for their optimum growth temperature around 24-26 ºC, with supraoptimal values above 30 ºC and suboptimal below 15-20 ºC [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 night-time biomass loss. In order to find out whether this correlation held a statistical meaning, the productivities versus the corresponding night-time biomass loss are graphically reported, combining every data point of both trials (Figure 3).

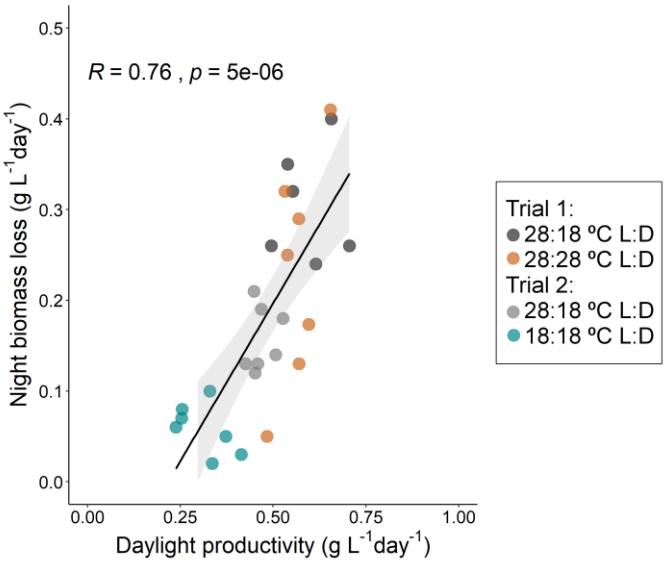

Figure 3 Correlation between daily yield and biomass loss, combining all data points from both trials using *Nannochloropsis oceanica* grown outdoors in tubular photobioreactors under different temperatures. Data points from the 28:18 ºC L:D (controls) cultures are depicted in dark (Trial 1) and light (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-value = 5×10^{-6}) 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 night-time biomass loss ($R(25)$=0.76, p<0.05). Linear relationships between biomass loss and growth rates have been reported before in nutrient-sufficient cultures kept in L:D cycles [8,32]. The high dispersion of the cells kept at 28:28 ºC reveals that at the constant temperature of 28ºC, night-

time biomass loss was more affected by temperature than by daily yield. On the other hand, the low dispersion of the cold points reveals that cells kept constantly at 18:18 ºC had their yield and night-time biomass loss inhibited by the cold temperature of 18 ºC. This reveals that temperature can affect night-time biomass loss as well as productivity, in spite of both these factors (night time biomass loss and daylight productivity) being correlated.

A summary of the daily biomass yields and the corresponding nocturnal biomass loss is shown in Supplementary Table S2. We are aware of the differences both in light energy amount and intensities that may have occurred between the two trials, except the length of the light period which was similar in both the trails (from 8 am to 6 pm). However, calculation of the growth yield (g/MJ), that is, by normalizing daylight areal yield (g/m^2/day) to the daily energy received by the cultures (MJ/m^2/day) in cultures grown according to the temperature regime of 28-18 °C L:D (control) resulted comparable between the first and second trial, 1.517 and 1.475 g/M, respectively, indicating that the light utilization efficiency of the cultures was very similar (Table S2), while that attained by the culture grown at the suboptimal temperature (18-18 °C, L:D) was much lower (0.987 g/MJ) indicating a clear effect of the low temperature on daylight growth (Table S2).

Daylight biomass productivity showed good correlation to light irradiance. Indeed, higher daylight yields 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 yield 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 yield 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 a 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 has been 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 slowing down the photosynthetic rate, inhibiting PSII repair mechanisms, and slowing down the protective mechanisms preventing photoinhibition [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.

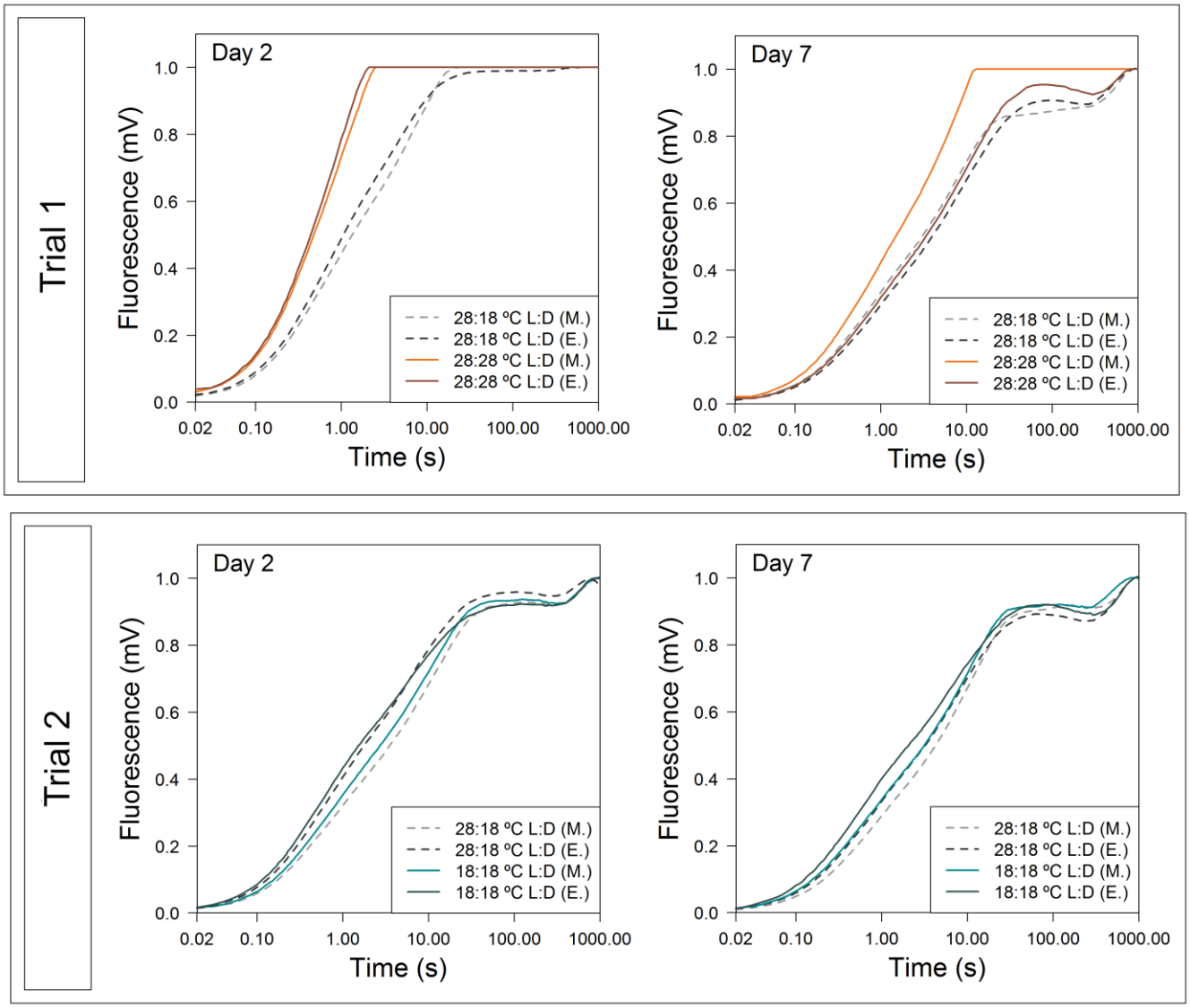

Figure 4 Kinetic representation of the OJIP transients from *Nannochloropsis oceanica* grown in tubular photobioreactors outdoors under semi-continuous regime and subjected to different temperatures. The first row shows the OJIP curves for the morning (M.; orange) and evening (E.; brown) samples of the 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}), we can see that the efficiency with which a PSII-trapped electron is transferred 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 in the beginning of the first trial due to the warmer temperatures overnight (28:28 ºC L:D) – but also became closer to 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 (Φ_{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 (Φ_{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 recovered, eventually reaching values closer to the control. Reaction centre densities (RC/CS_m) were a bit lower than the control but 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, 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 in time of the fraction of Q_A reduction [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 and then recovered towards the end. Nonetheless, the cells 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).

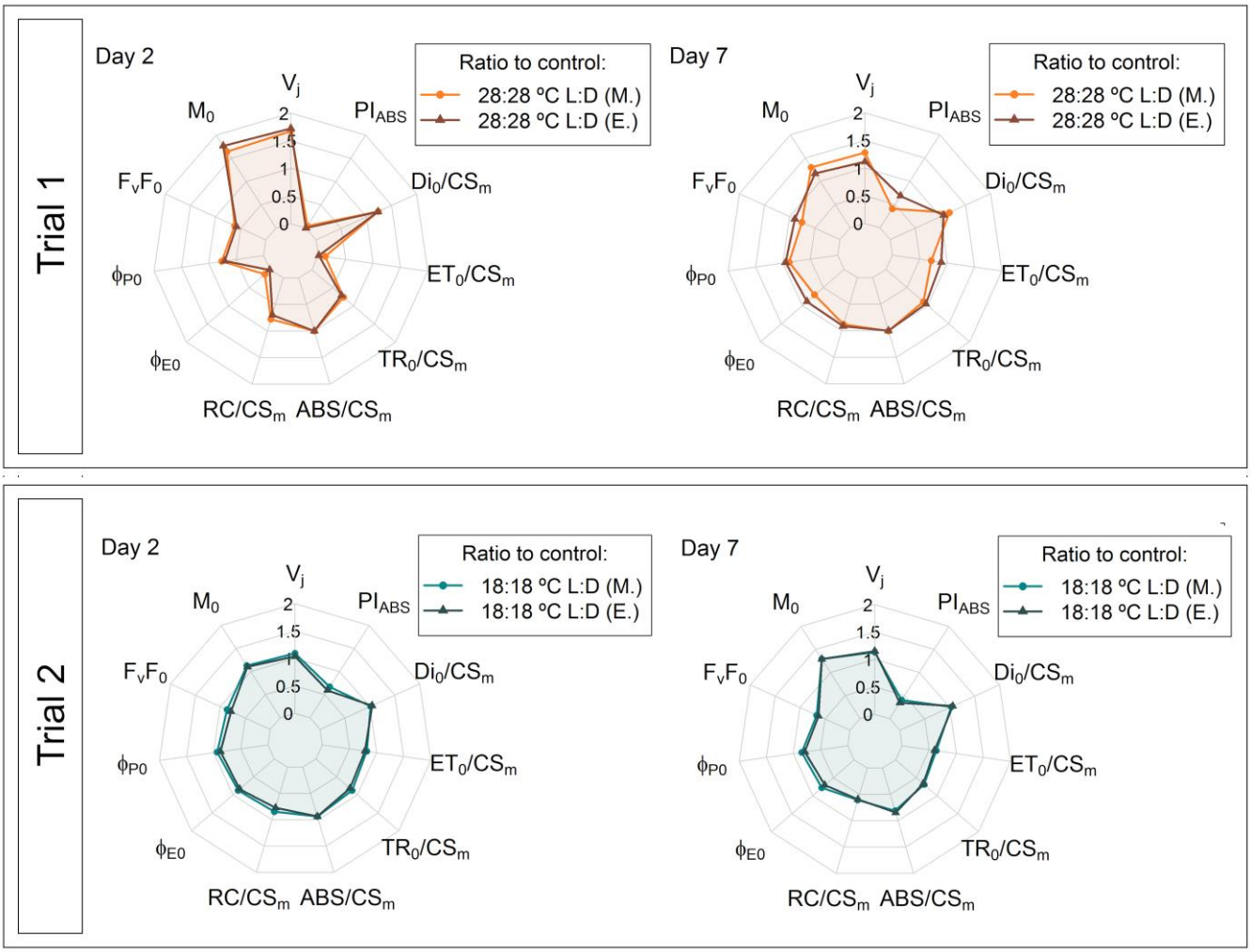

Figure 5 Temperature influence on OJIP determined parameters of *Nannochloropsis oceanica*, grown in tubular photobioreactors outdoors under semi-continuous regime and subjected to different temperatures. Spider plots depict the ratio of the conditions (28:28 ºC L:D in the first trial, and 18:18 ºC L:D 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 oxygen evolution and respiration measurements

The relation found between respiration and net photosynthesis lies within the expected values [8], but the correlation between night-time biomass loss and respiration values by the end of the dark period (morning) found using all data points revealed a high dispersion (R = 0.64, p = 0.00017; 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 in both conditions.

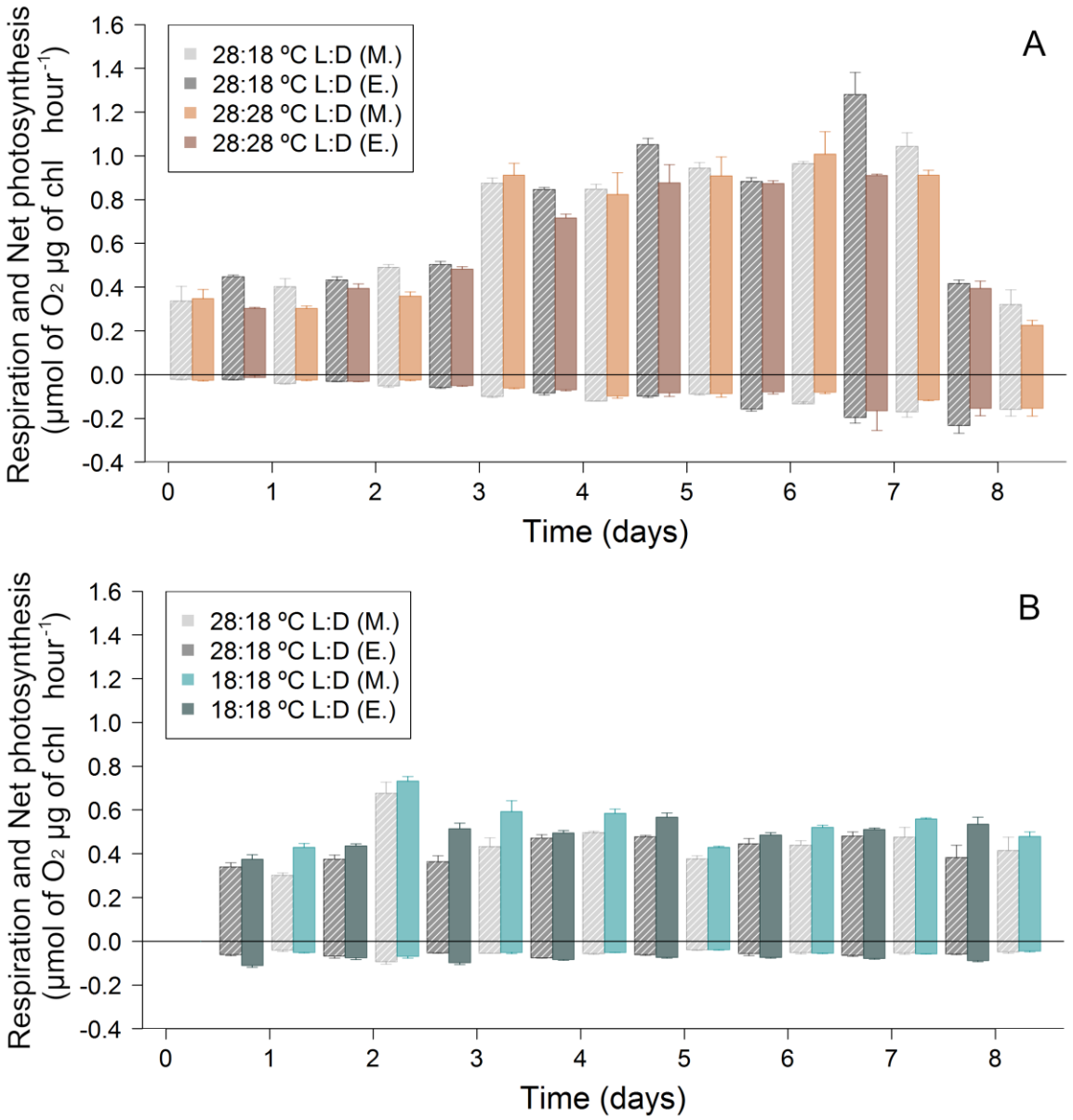

Figure 6 Net photosynthetic oxygen evolution (y>0) and respiration (y<0), normalized to chlorophyll of *Nannochloropsis oceanica* grown in semi-continuous mode in outdoor tubular photobioreactors at different temperatures, showing Trial 1 (A) and Trial 2 (B). Values for cells grown at 28:28 ºC L:D or 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.

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) 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 allowed the cell to recover photosynthesis 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 transfred to 28 °C, indicating that photosynthetic activity can recover very fast.

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.

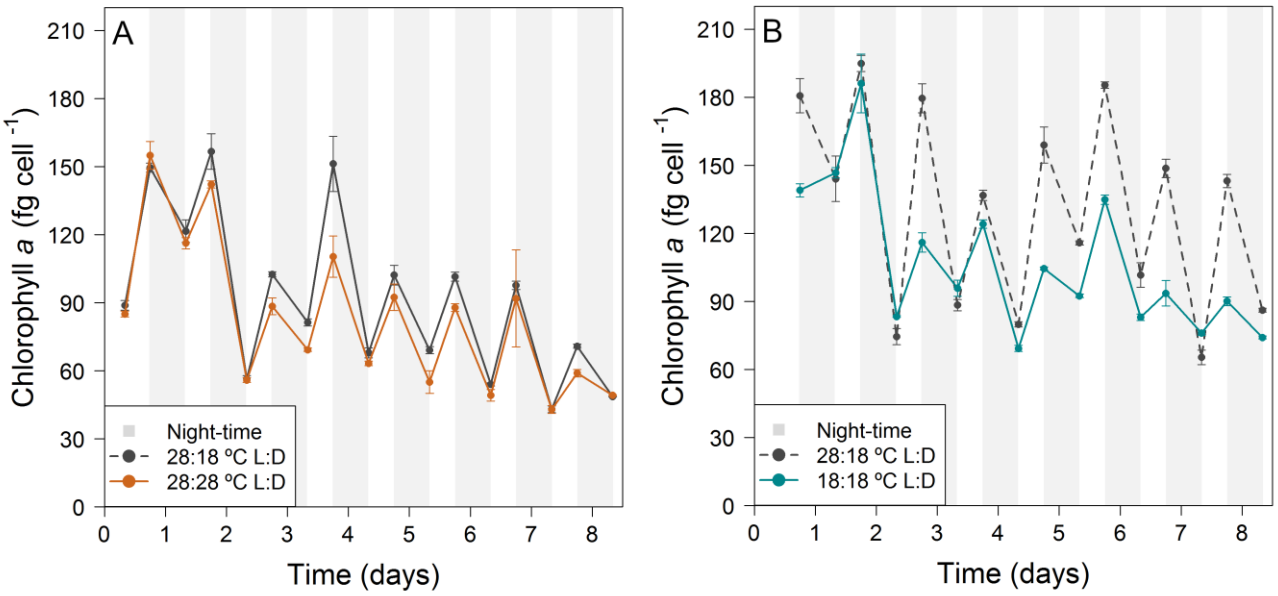

Figure 7 Chlorophyll a content per cell of *Nannochloropsis oceanica*, grown in outdoor tubular photobioreactors under semi-continuous regime and subjected to different temperatures, showing Trial 1 (A) and Trial 2 (B). Grey lines represent the controls when cells were kept at 28:18 ºC L:D, and orange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

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, since 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* revealed 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).

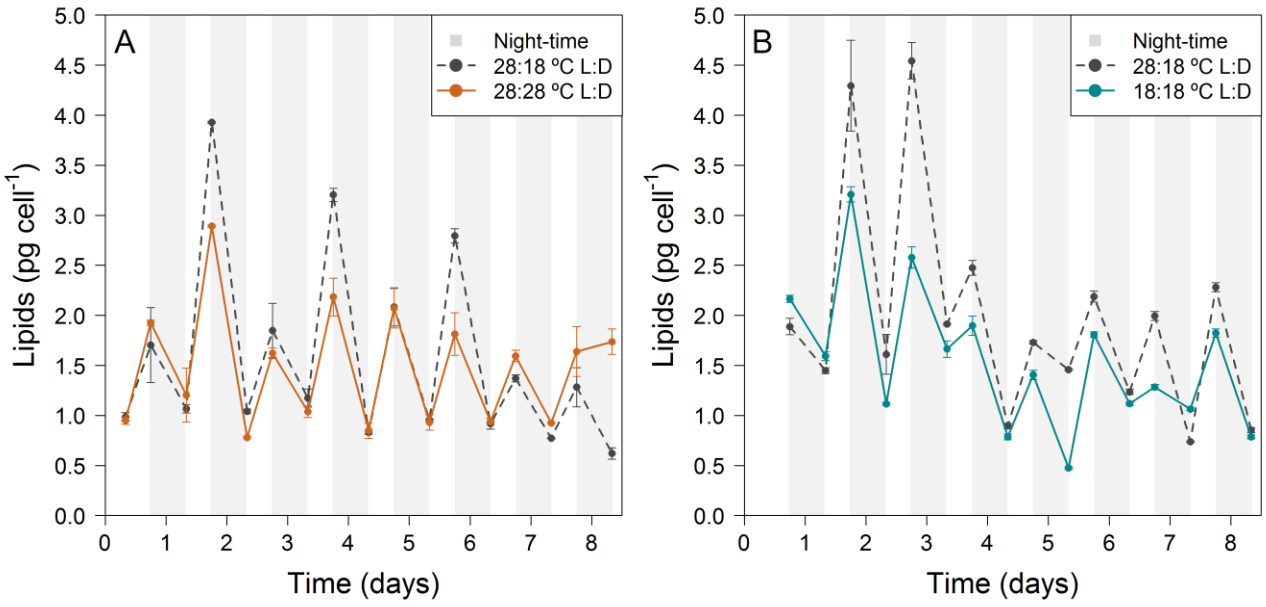

Figure 8 Temperature influence on the lipid content of *Nannochloropsis oceanica* cells, grown in tubular photobioreactors outdoors under semi-continuous regime and different temperature profiles. In Trial 1 (A) the culture was kept at 28:28 ºC L:D (orange line), and in Trial 2 (B) the cells were kept 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, however 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 at 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 the tested growth conditions. 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 percentage in the biomass of *N. oceanica*. The increase of polyunsaturated fatty acids (PUFA), at the expense of SFA, under lower temperature conditions is in accordance 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 night-time to generate the necessary ATP levels for the cell to survive during the dark period, synthesize DNA, and enter into 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.

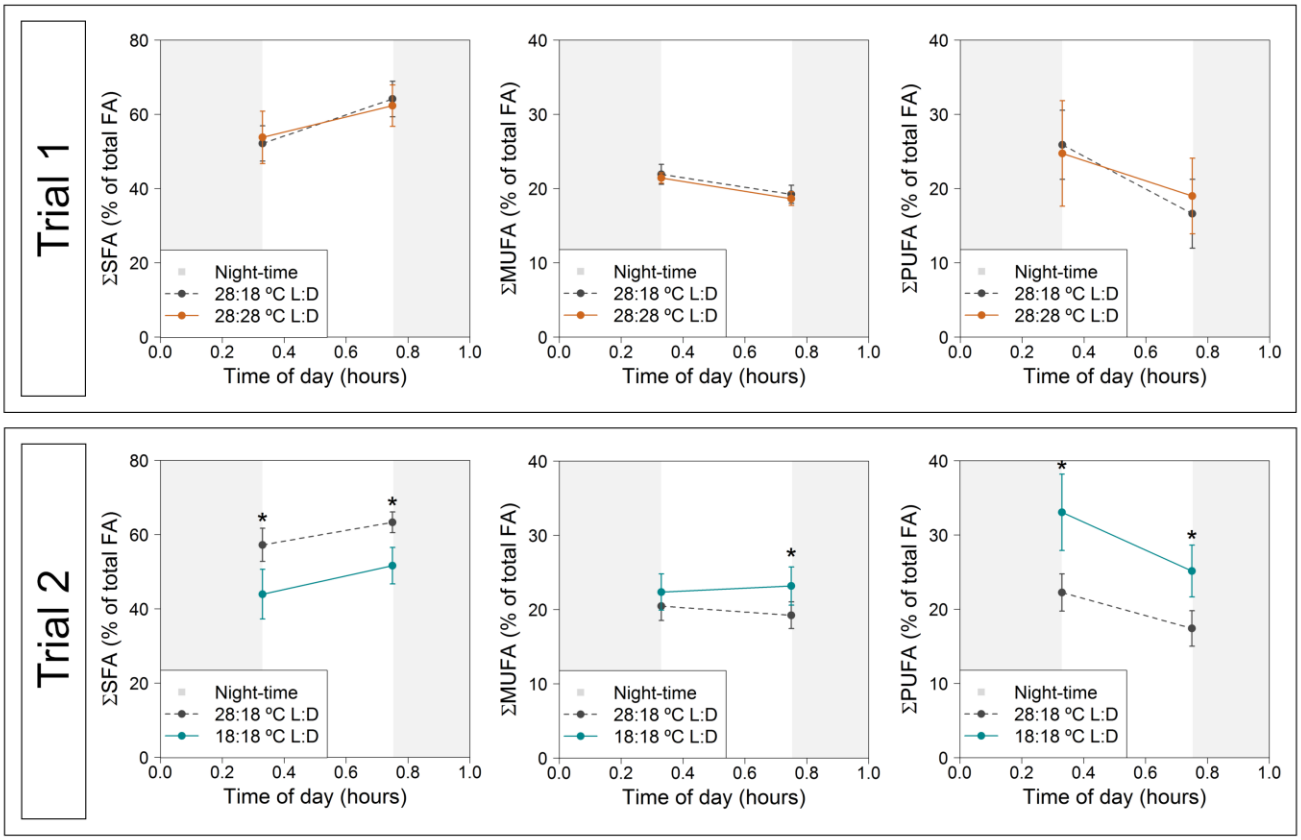

Figure 9 Daily average (± SD) of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) for morning and afternoon samples of *Nannochloropsis oceanica*, grown in a semi-continuous cultivation mode in tubular photobioreactors outdoors under different temperature 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 showed a minor influence of temperature upon this ratio in *N. salina* [25].

4. Conclusions

Contrary to what one could expect, higher night biomass losses were observed especially for the cultures operated at dual temperatures of 28 °C during daytime, and 18 °C during night-time. 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 night-time biomass loss, in cultures operated in a semi-continuous regime. However,

this relation can be affected 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 during the night. 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 mass 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, which 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 study. 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, FXM performed a critical revision. Funding for the experiment and analysis was supported by GCZ, JV, FXM and GT. 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 night-time 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 night-time 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. Night-time 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

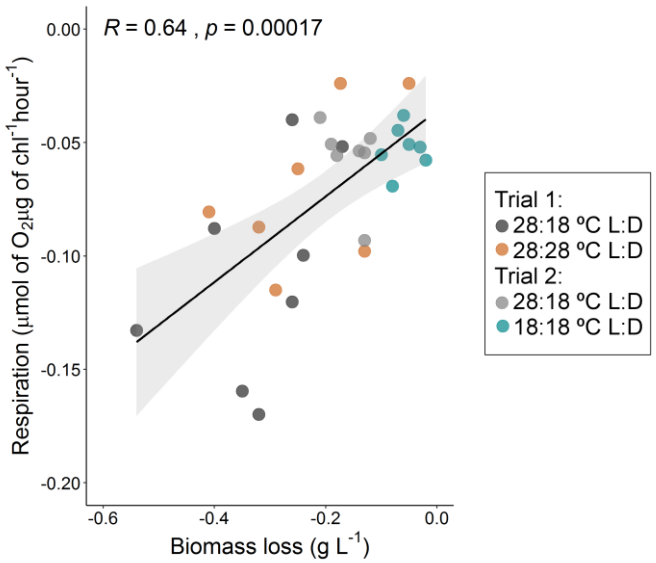
Time (days)	**Daily volumetric productivity (g L^{-1} d^{-1})**		**Daily volumetric night-time biomass loss (g L^{-1} d^{-1})**		**Night-time biomass loss (%)**	
	28:18 °C (L:D)	28:28 °C (L:D)	28:18 °C (L:D)	28:28 °C (L:D)	28:18 °C (L:D)	28:28 °C (L:D)
0	0.496 ± 0.111	0.596 ± 0.061	0.260 ± 0.125	0.173 ± 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 night-time biomass loss (g L^{-1} d^{-1})**		**Night-time 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 biomass yields (gross and net) and night-time biomass loss (± SD) of *Nannochloropsis oceanica* cultures grown under different temperature regimes in tubular photobioreactors (PBR) outdoors in two independent trials. Gross yield (g m^{-2} d^{-1}) was determined by dividing the daily average biomass produced (g d^{-1}) with the PBR's area (m^2). Net yield (g m^{-2} d^{-1}) was determined by subtracting the gross yield with the night biomass loss (g m^{-2} d^{-1}). The night-time 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	Gross yield (g m^{-2} d^{-1})	Net yield (g m^{-2} d^{-1})	Night biomass loss (g m^{-2} d^{-1})
1	28:18 °C L:D	30.8 ± 5.53	14.5 ± 7.81	16.4 ± 5.52 (53.1%)
	28:28 °C L:D	27.8 ± 4.11	16.6 ± 7.13	11.2 ± 5.82 (40.3%)
2	28:18 °C L:D	24.2 ± 1.68	16.1 ± 2.39	8.10 ± 1.69 (33.5%)
	18:18 °C L:D	16.2 ± 3.21	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*-value = 1.7 × 10^{-4}) are shown in the upper left corner of the plot (n=29).