Annual outdoor cultivation of the diatom *Thalassiosira weissflogii*: productivity, limits and perspectives

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ABSTRACT

Diatoms are a promising source of renewable biomass for production of energy, functional ingredients and food products. The eurythermal diatom *Thalassiosira weissflogii* shows great resistance and robustness, and can be considered a suitable candidate for outdoor cultivation. In this study we investigated the resilience of one strain of this species during one-year outdoor experimentation in inclined tubular photobioreactor with a working volume of 40 L. Cells of *T. weissflogii* overcome temperature and irradiance fluctuations by adapting their growth rate at the different environmental conditions. Calculation of areal biomass productivity based on 30 cultures along the year showed a median value of 3.83 g m^{-2} day^{-1} with maximal values of biomass (6.8 g m^{-2} day^{-1}) and lipids (1.2 g m^{-2} day^{-1}) reached in February and July, respectively. We observed a decline in biomass productivity with temperature increase, whereas lipid content (10-20% of dry biomass) was not significantly affected by the environmental conditions as far as a high concentration of nutrients was maintained. The outdoor experimentation successfully proved one year-round production of biomass from a single diatom species in closed photobioreactor, making this diatom species a suitable candidate for guaranteeing stable annual productive cycles in outdoor plan.

Keywords (6 words): microalgae; biomass; biofuels; productivity; inclined tubular photobioreactor; outdoor experimentation.

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1. Introduction

In the last decades, microalgae have attracted a great deal of attention since they convert solar energy and CO_2 into biomass. Microalgae cultivation do not compete for land with terrestrial crops, and could contribute to mitigate carbon dioxide and other pollutants [1,2]. Microalgal biomass can be used without fractionation (algal flour) or can be exploited as source of a great number of commercially interesting bioproducts such as biofuels, pigments, cosmetics, drugs and food ingredients [3-11].

In the last 50 years, large-scale cultures of microalgae for commercial purposes have been achieved in open ponds and photobioreactors in outdoor facilities [12-14]. The choice of the cultivation system affects biomass productivity and depends on intrinsic factors such as algal biology, land and energy cost, irradiance, temperature and other environmental conditions. Although the use of photobioreactors (PBRs) requires higher capital costs, they are considered more effective than open ponds to curtail evaporation and contamination risks.

Currently, large-scale microalgae cultivation include selected microalgal strains (e.g. *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella* and *Spirulina* genus, *Nannochloropsis* spp., *Isochrysis galbana*, *Pavlova lutheri*, *Tetraselmis spp*., *Phaeodactylum tricornutum)* mainly for acquaculture, and as sources of β-carotene, astaxanthin, nutritional supplements, EPA and DHA [13, 15-19].

Among microalgae, diatoms (Bacillariophyceae) have received a great deal of attention for their potentiality in the development of sustainable biobased processes [20-23]. This is mainly due to their high duplication rates, the plasticity of carbon allocation within cellular components, the significant resiliency to environmental changes, high sedimentation rates that could considerably reduce the harvesting costs. The major challenge to propose diatom-based scalable bioprocesses is the evaluation of the sustainability of cultivation cycles in outdoor facilities of selected robust diatom strain [24-26]. Most of the data about annual biomass productivity of these microalgae are so far based on extrapolation of laboratory data, indoor experimentation or short periods of outdoor cultivation [19, 27-30].

Recently, the diatom *Thalassiosira weissfloggi* was selected as a productive microalgal strain, able to respond to different stimuli (e.g nutrient input and CO_2 insufflation) modulating biomass content and distribution of biochemical components within the cell [31-33].

Here we report the results of one-year cultivation of the marine diatom *T. weissflogii* in an outdoor inclined photobioreactor. The main aim of this study was to test resistance of this diatom to unsterile conditions and environmental variations, as well as to prove one year-round diatom cultivation of a single strain. Furthermore, this study gave us the opportunity to assess changes in growth rates in response to temperature and light fluctuations, and to make an estimation of biomass productivity based on the data obtained along the year.

2. Materials and Methods

2.1 Microorganisms and culture conditions

Thalassiosira weissflogii (P09) is a local strain, isolated from the Gulf of Naples, Italy (latitude 40° 43′ 90″ N; longitude 14° 10′ 167″) Cells were grown in F/2 medium [34] prepared in oligotrophic sea water (salinity 36-38 g kg^{-1}) filtered at 20 µm and buffered at pH 8 by 400 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIZMA®, Aldrich).

2.2 Outdoor pilot-scale photobioreactor

T. weissflogii was grown outdoor under batch modality in a transparent poly-methyl methacrylate (PMMA) photobioreactor (M2M Engineering, Naples, Italy) with a tubular working volume of 40 L (2.2 m x 0,2 m) from July 2009 to July 2010. The apparatus was installed in the experimental area of CNR in Pozzuoli - Naples (40°49’40”N; 14°70’30”E). The cylindrical culture chamber was oriented from North to South, with an angle of approximately 27° on the plane. Cultures were mixed-up by air sparging (5 L/ min) through three perforated pipe spargers running along the lower side of the culture chamber (hole size diameter: ca 10-15 µm) and a large diffuser tube (hole diameter: 33 mm) located at the bottom of the culture chamber (Figure 1, Supplementary Material).

Inocula for the photobioreactor were all taken from laboratory axenic cultures in healthy exponentially growing phase, and diluted with seawater up to an initial cell density of 9000-15000 cell mL^{-1}. *T. weissflogii* cultures in photobioreactor were supplemented with nutrients contained in F/2 medium every day ($NaNO_3$ 75 mg L^{-1}, $NaH_2PO_4.H_2O$ 5 mg L^{-1}, $Na_2SiO_3.9H_2O$ 30 mg L^{-1}, trace metal solution 1 mg L^{-1} culture, vitamin solution 0.5 mg L^{-1} culture). Growth was measured by daily cell count with a Bürker counting chamber (Merck, Leuven, Belgium, depth 0.100 mm) under an inverted microscope (Nikon Eclipse TE200, Nikon Corp., Tokyo, Japan) at 200x magnification.

Temperature inside the cultivation chamber was measured twice daily by an external probe.

Ambient temperature during the cultivation period is available at http://www.agricoltura.regione.campania.it/meteo/riepiloghi_2009.html and http://www.agricoltura.regione.campania.it/meteo/riepiloghi_2010.html

2.3 Biomass collection

Growth curves have different duration in response to the climatic conditions. At the fisrt point of declining phase for each growth curve, one litre of culture was collected and harvested by centrifugation in a swing out Allegra 12XR (Beckman Coulter Inc., Palo Alto, CA, USA) at 2300 *g* (4°C) for 10 minutes. Pellets were washed twice by 0.5 M ammonium formate solution [35] to remove salts and stored at -20°C. Biomass was dried by lyophilization (MicroModulyo, Thermo Fisher Scientific). Total biomass dry weight (mg L^{-1}) was determined gravimetrically on lyophilized pellet. Biomass productivity was estimated according to the formula:

$$\text{Productivity}_i = \frac{W_i\,(t_{\text{end}}) - W_i\,(t_0)}{t_{\text{end}} - t_0}$$

where the symbols stand for:
W=weight of biomass
t = time (days).

2.4 Chemical analysis

Lipid extraction was performed using the modified Folch method on lyophilized cell pellet [36]. Lipid content (mg L^{-1}) was determined gravimetrically. Lipid productivity was estimated according to the formula:

$$\text{Productivity}_i = \frac{L_i\,(t_{\text{end}}) - L_i\,(t_0)}{t_{\text{end}} - t_0}$$

where the symbols stand for:
L=weight of lipid extract
t = time (days).

Lipid extracts were dissolved in 700 µl $CDCl_3/CD_3OD$ 1:1 (v/v) and transferred to the 5-mm NMR tube. Chemical shift was referred to CHD_2OD signal at δ 3.34. ^{1}H-NMR spectra were recorded on Bruker DRX 600 spectrometer (Bruker, Germany). Diagnostic signals at 2.35 ppm (allylic protons, 2 signals for each unsaturated fatty acids) and at 2.06 ppm (methylene protons α to carboxy group, total fatty acids) were integrated in the ^{1}H-NMR spectra. Percentage distribution between saturated and unsaturated fatty acids was estimated according to the following formulas:

$$\%\text{ unsaturated fatty acids} = \frac{\text{Area allylic protons}/2}{\text{Area protons } \alpha \text{ to carboxy group}} * 100$$

$$\%\text{ saturated fatty acids} = 100 - \%\text{ unsaturated fatty acids}$$

2.5 Statitistical analysis

The relationships between temperature, irradiance and biomass productivity were tested using linear regression analysis with a confidence intervals equal to 95%. Linear regressions significance were calculated using the Fisher test. The statistical calculation software was Graphpad Prism 6.0 version.

3. Results and Discussion

3.1 Cell growth and environmental factors

The marine diatom *Thalassiosira weissflogii* was grown in 40-L, N-S oriented, 27°-inclined tubular photobioreactor., for year-round under batch modality. The experimental site is located in the Southern Italy and it is subjected to the typical Mediterranean climate characterized by mild, wet winters and warm to hot, dry summers. During the year of experimentation (July 2009-June 2010), ambient temperature in summer and spring ranged from 10°C to 35°C, whereas in autumn and winter temperatures ranged from a minimum value of 0° C to a maximum value of 25°, being January, February and March the coldest months (Figure 1). The temperature detected inside the culture chamber ranged from a maximum value of 42°C in July to a minimum value of 2° C in February and January. Solar irradiance varied in the range from 6 MJ m^{-2} in winterly months to 22 MJ m^{-2} in warmer months. Cells were cultivated under batch modality in non sterilized sea water for a whole year. Cultures were inoculated with exponentially growing cells in order to have a starting point of 9000-15000 cell mL^{-1} cell mL^{-1}. Based on nutrient consumption rate recently reported in *T. weissfllogii* [33], each batch was maintained under high nutrient regime sustained by daily supplementation of macro and micro-nutrients in the quantity expected in the F/2 recipe. Growth curves were followed every day and cells were harvested at the first point of the declining phase for the evaluation of biomass and lipid production. In support of the positive response of *T. weissflogii* to the varying environmental conditions, no culture failed even if significant differences were recorded in terms of growth curve duration (Figure 2). Cells rapidly increased growth rate in summer and early autumn. From July to October, growth curves lasted 3-4 days, reaching a maximum cell density of 280000-300000 cells mL^{-1}. During the winter, cultures entered into stationary phase in 6-12 days. The slowdown of growth rate was counterbalanced by the increase of cell concentrations, which reached,in January, densities of 500000 cells mL^{-1}. In comparison to summer batches, winter cultures also showed a most elusive shift from late exponential/early stationary phase to the senescent phase (Figure 2, Supplementary Material).

Temperature and light are the most important factors that affect microalgal growth in nutrient-sufficient culture conditions [37-39].

Excessive photonic energy is usually dissipated as heat [40], and could limit or inhibit growth, especially when also air and culture temperature are high. Hence, we can hypothesize that combined effects of temperature and irradiance could be more detrimental in summer than in other seasons, and that temperature could be accounted as the main physical factor governing algal growth and the rate of metabolic reactions [41].

Several authors have reported that temperature control is a key factor for the microalgal cultivation in outdoor systems [42-43]. Optimal temperature of growth and resilience to seasonal temperature fluctuations are an intrinsic characteristic of microalgae and are usually species-specific [44]. Commonly, microalgae tolerate temperatures between 16 and 27 °C. Temperatures lower than 16°C slow down the growth rates growth, whereas those higher than 35°C are lethal for a number of species. Previous studies showed that growth rate of *T. weissflogii* increased linearly with temperature in the range 12-20°C and decreases above and below this range.[45].

Outdoor cultivation of microalgae, such as Chlorella and Scenedesmus genus, require additional devices to control temperature around the optimal value of 20-25°C [42]. Unfortunately, temperature control units are generally energy expensive process, especially for managing a huge volume of culture media. Although no temperature control has been associated to the inclined tubular photobioreactor, cells of *T. weissflogii* were able to face temperature fluctuations adapting their growth rate.

3.3 Biomass and lipid production

Biomass production was evaluated at the first point of declining phase for each batch culture. Assessment of biomass production revealed a range from 150 to 250 mg L^{-1} in summer months (Figure 3). This value increased with the reduction of the growth rate. Thus, a 5-fold increase of biomass quantity occurred in winter, with values ranging from 300 to 1000 mg L^{-1}. Statistical analysis showed a good linear correlation between cell density and biomass production along the whole experimental period (R=0.605; P=0.0001). In agreement with this correlation, the major biomass production was achieved when cell concentration in diatom cultures was higher than 300000 cell/ ml. Lipid production was estimated by gravimetric analysis after lipid extraction of biomass with organic solvents. As shown in Figure 3, the overall lipid content was consistent with the biomass changes during the annual study, in agreement with a constant percentage of lipid components in the diatom cells, ranging from 10 to 20% of total dry biomass. Despite the fluctuations of the environmental conditions and their potential stress on lipid accumulation process in diatom cells, the effect of high nutrient regime maintained by a daily nutrient input seems to predominate on other climatic factors in maintaining a constant lipid percentage along all the experimental period. Data are in agreement with those obtained in laboratory cultures of *T. weissfloggii* grown under the same conditions, in which high nutrient regime determine a lipid percentage of 20% of total dry biomass [33]. Many approaches were described in laboratory studies to increase lipid level respect to the other main biochemical components, eg. nutrient deprivation, CO_2 insufflation [33, 46-48]. In the case of a customization of diatom biomass for bio-oil exploitation, lipid content should further improved by tuning nutrient input (e.g. silicate limitation) using different cultivation schemes.

Distribution of total fatty acids between saturated and unsaturated fatty acids was assessed by ^{1}H-NMR analysis on lipid extracts. Unsaturated fatty acids always predominated on saturated components, with values ranging between 50% and 80% (Figure 3, Supplementary Material). Generally microalgal cells adapt to live at different temperatures modulating the membrane fluidity, in terms of compositions of fatty acids in membrane lipids [49]. During our study, the fluctuations in fatty acid unsaturation are not directly correlated to temperature variations, probably due to the fact that cells were cultivated under batch conditions and don't have sufficient time to acclimate at different temperatures for prolonged periods.

3.4 Assessment of annual productivities

Annual biomass productivity showed an average value of 60 mg l^{-1} day^{-1} (Figure 4A). Biomass increase was concomitant with the increase of batch cultures duration in the winter months. As a consequence, biomass productivity was higher than the annual average from February to May, and below or near to the medium value in the other months. A reduction in biomass productivity, therefore, occurred with increasing duration of exposure to high or low temperature stress (Figure 4A). Linear regression analysis highlight that the biomass productivity showed a temperature dependence and no significant relationship with irradiance. Particularly, the 38% of the biomass increment is ascribable to the decrease of mean temperature (Table 1, Supplementary Material).

Lipid productivity varied from 5 mg/l day to 13 mg/l day, with an average annual value of 7.5 mg L^{-1} day^{-1} (Figure 4A, Supplementary Material).

Productivity values are in line with those reported for laboratory cultivation of *T.weissfloggii* under the same conditions of nutrient input [33] suggesting that, in our case, the scale-up of the cultivation system from 2L to 40 L and the exposure of the cells to environmental fluctuations of temperature and irradiations didn't affect the global balance of biomass productivity on annual scale.

In this study, areal biomass productivity was calculated considering the area occupied by the photobioreator and the biomass productivity values obtained from 30 cultures performed in a whole year. Areal annual biomass productivity ranged from 1 to 6.8 g m^{-2} day^{-1} over year-round experimentation, with an interquartile range from 2,7 and 4.7 g m^{-2} day^{-1} and a median value of 3.83 g m^{-2} day^{-1} (Figure 4B).

These values were lower than the calculated threshold of 17 g m^{-2} day^{-1} required to achieve a positive Energy Return On Investment (EROI) for microalgae cultivation. On the other hand, only few studies really estimate the productivities of diatom-based outdoor cultivation systems, reporting values lower than 10 g m^{-2} day^{-1} in line with other oleaginous microalgae [29].

Although the median values of areal productivity were lower than the estimated potential for diatoms (from 8 to 39 g m^{-2} day^{-1} [21]), this is the first report based on outdoor experimentation for an year-round on a single diatom species. Most of the data about annual biomass productivity of diatoms are so far based on indoor experimentation or outdoor cultivation for few months over the year of [19, 22, 27-30]. The only study of a one-year outdoor cultivation of diatoms suggested to alternate warm- and cold- tolerant species to guarantee a year-round biomass and lipid production, but did not identify a single species able to survive to high temperature and irradiance fluctuations [29].

The data collected over the year along the outdoor experimentation on *T. weissflogii* cells underlined its great resistance and robustness, that is also strengthened by its cosmopolitan distribution in temperate and cold aquatic zones all over the word [50]. Its intrinsic capacity to adapt and live in different environments is thus particularly suitable for outdoor experimentations, especially in areas with strong climate variations. Resilience of *T. weissflogii* can be used to develop outdoor bioprocess based on the exploitation of this species for biomass production for different applications, such as biofuel production, as food & feed, production of drugs, cosmetics, fertilizers.

In the complex, the study underlines the great technological advantage for using *T. weissflogii* in microalgae-based plan for biomass production, in a perspective of guaranteeing stable annual productive cycles in outdoor facilities.

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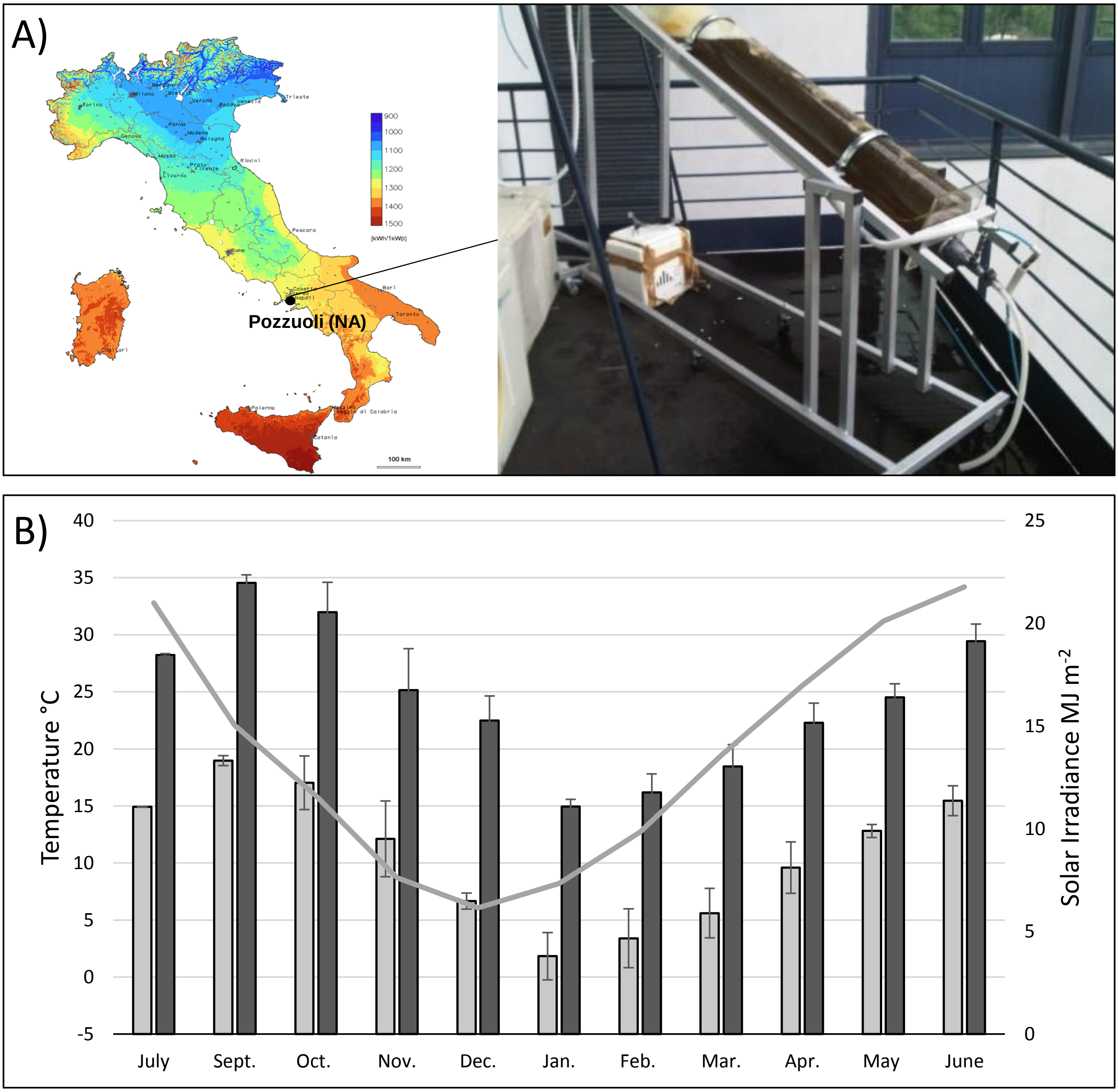
Figure captions

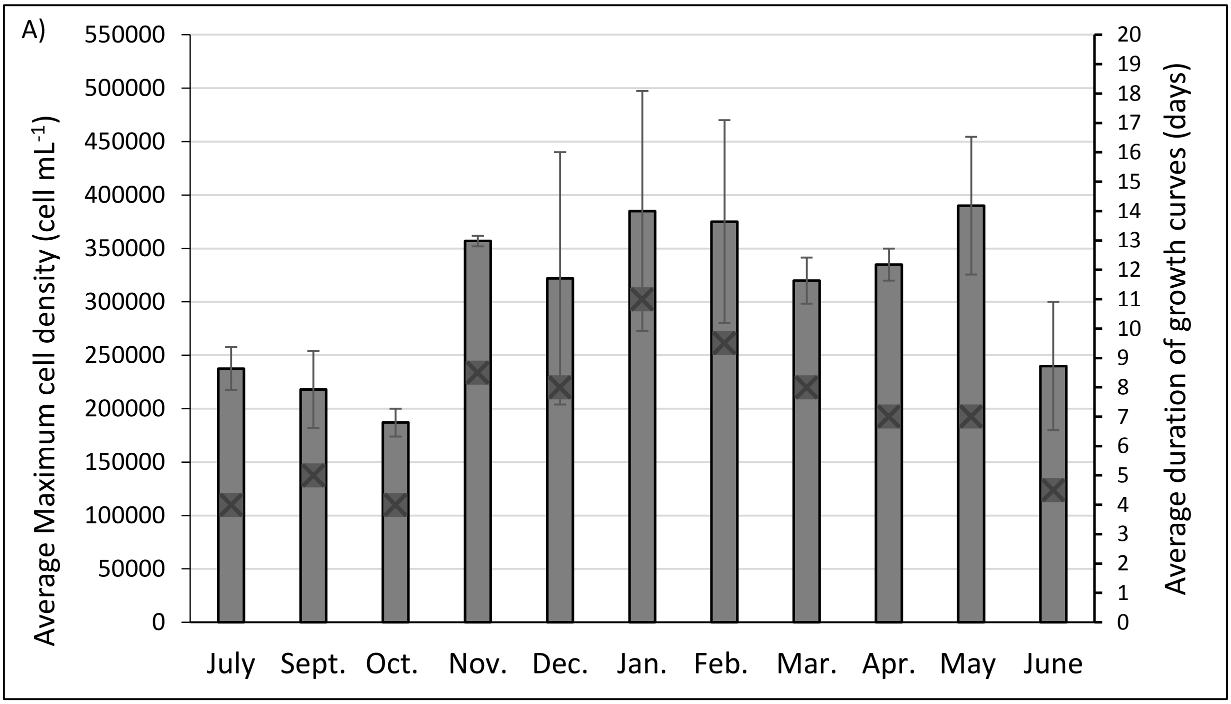
Figure 1: A) Location of experimental site and photo of inclined tubular photobioreactor (40 L) used for outdoor cultivation of diatom *Thalassiosira weissflogii*. B) Monthly average minimum temperature (light grey band) and maximum temperature (dark grey band). Solar irradiance (grey line), expressed as MJ m^{-2}.

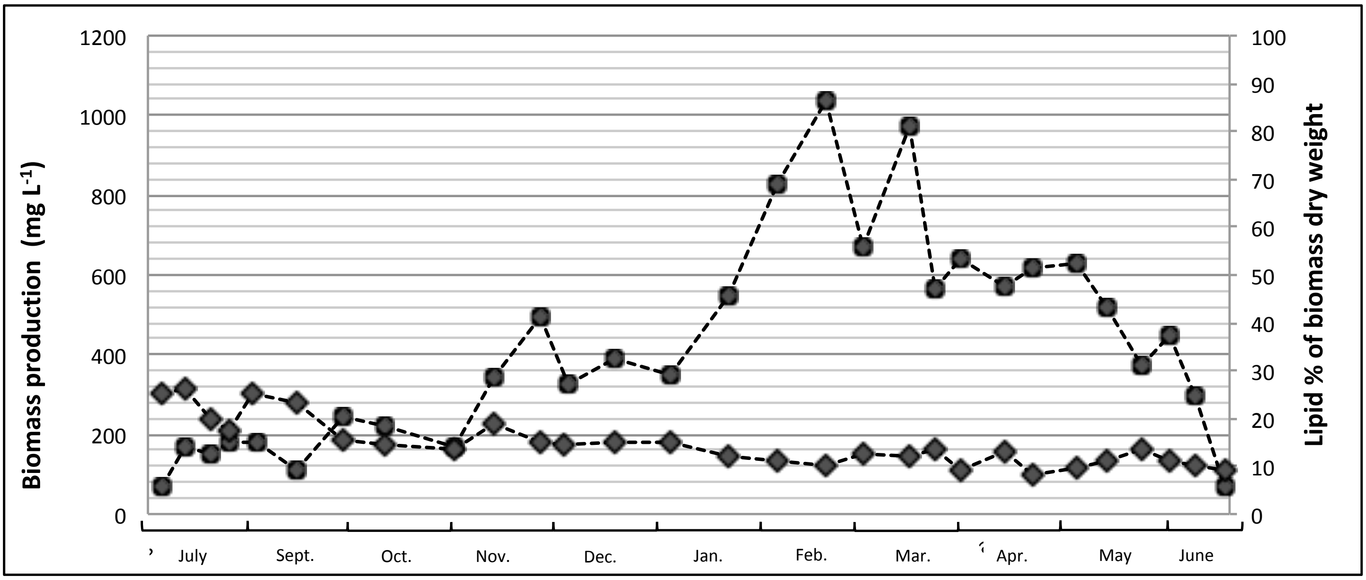
Figure 2: Average maximum cell density (expressed as cell ml^{-1}) obtained by *Thalassiosira weissflogii* cells cultivated under batch modality in 40 L inclined tubuar photobioreactor. The symbol x is referred to the montly average maximum duration of growth curves (days, secondary axis)

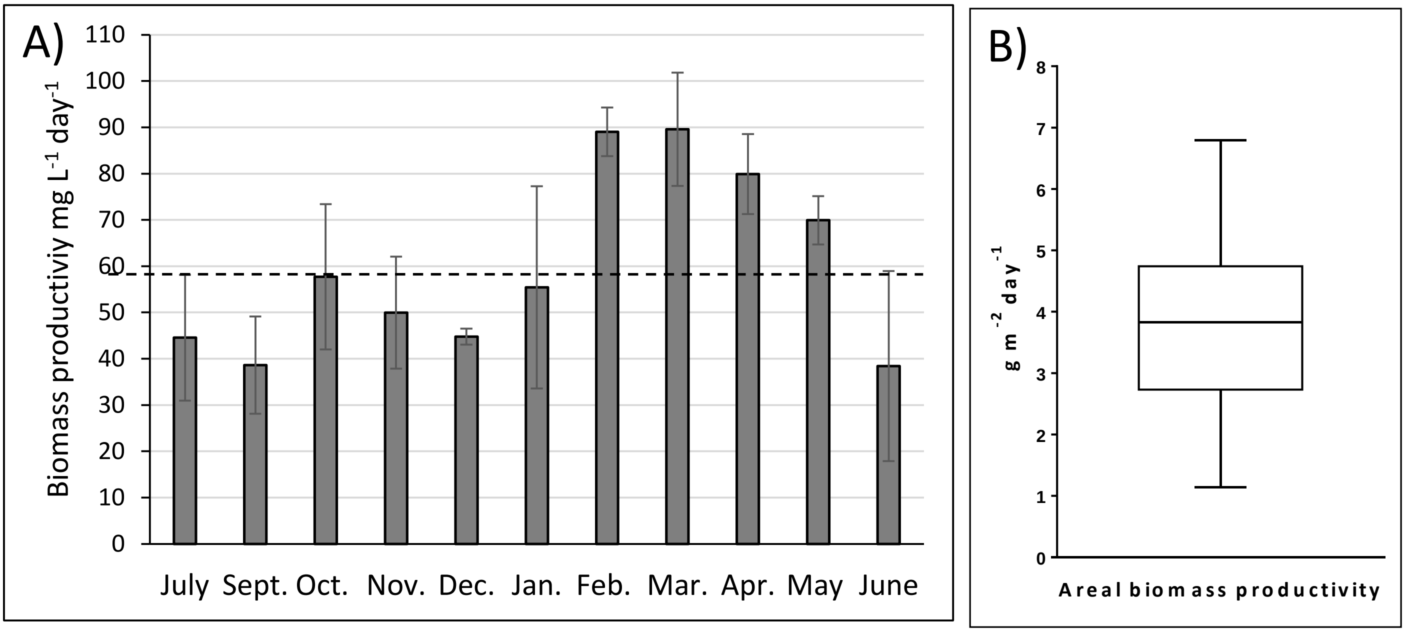
Figure 3: Dry biomass production, expressed as mg L^{-1} (dark bullet); lipid content, expressed as percentage of biomass dry weight (grey diamond, secondary axis).

Figure 4: (A) Monthly average biomass productivity, expressed as mg L^{-1} $day^{-1.}$ The dashed line represent the average value of annual biomass productivity (60 mg L^{-1} day^{-1}); (B) Boxplot of areal annual biomass productivity, expressed as g m^{-2} day^{-1}.


A)
Torino
Milano
Brescia
Verona
Padova
Venezia
Trieste
Parma
Modena
Bologna
Genova
Rimini
Massa
Prato
Firenze
Livorno
Pescara
Roma
Caserta
Napoli
Bari
Taranto
Cagliari
Palermo
Messina
Reggio di Calabria
Catania
Pozzuoli (NA)
900
1000
1100
1200
1300
1400
1500
[kWh/1kWp]
100 km
B)
Temperature °C
Solar Irradiance MJ m-2
40
35
30
25
20
15
10
5
0
-5
25
20
15
10
5
0
July
Sept.
Oct.
Nov.
Dec.
Jan.
Feb.
Mar.
Apr.
May
June


A)
Average Maximum cell density (cell mL^{-1})
Average duration of growth curves (days)
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
July
Sept.
Oct.
Nov.
Dec.
Jan.
Feb.
Mar.
Apr.
May
June


Biomass production (mg L-1)
Lipid % of biomass dry weight
1200
1000
800
600
400
200
0
100
90
80
70
60
50
40
30
20
10
0
July
Sept.
Oct.
Nov.
Dec.
Jan.
Feb.
Mar.
Apr.
May
June


A)
Biomass productiviy mg L^{-1} day^{-1}
0
10
20
30
40
50
60
70
80
90
100
110
July
Sept.
Oct.
Nov.
Dec.
Jan.
Feb.
Mar.
Apr.
May
June
B)
g m^{-2} day^{-1}
0
1
2
3
4
5
6
7
8
Areal biomass productivity

All the authors mutually agree that the manuscript should be submitted to *Algal Research.*
All the authors declare that there is not conflict of interest
The manuscript is the original work of the authors.

Looking forward to hearing from you,

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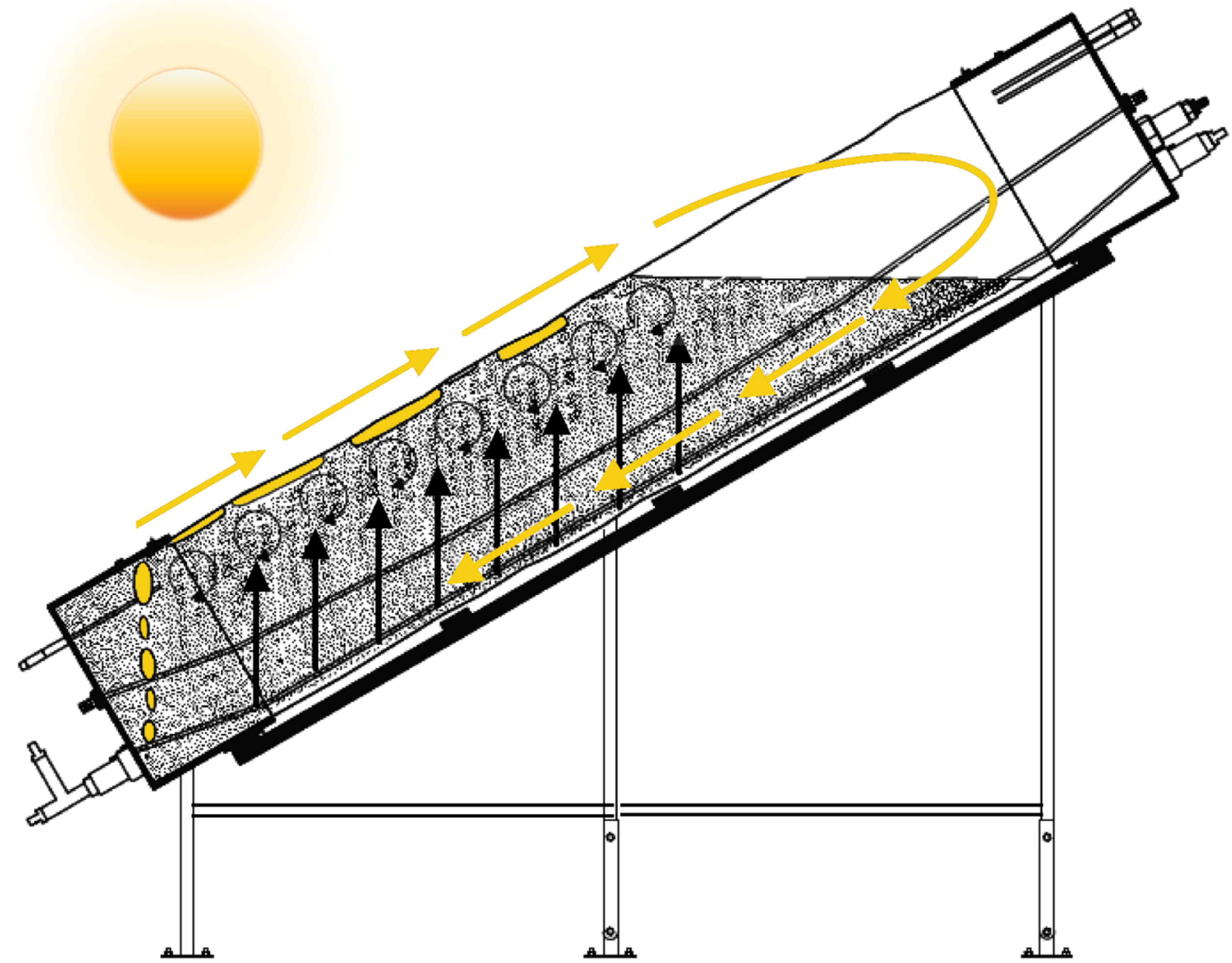
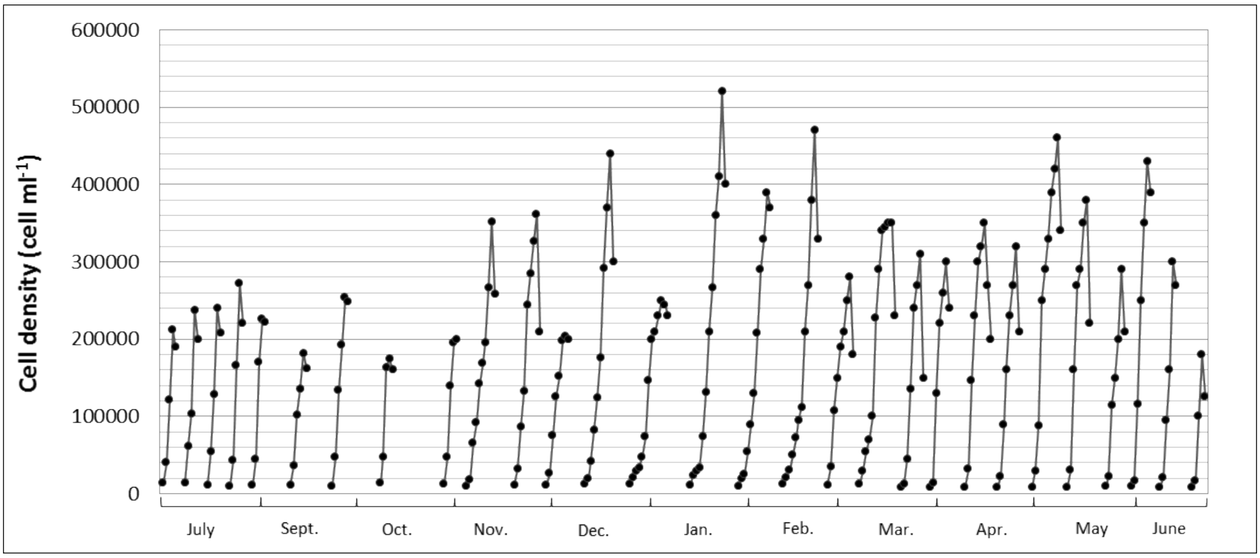
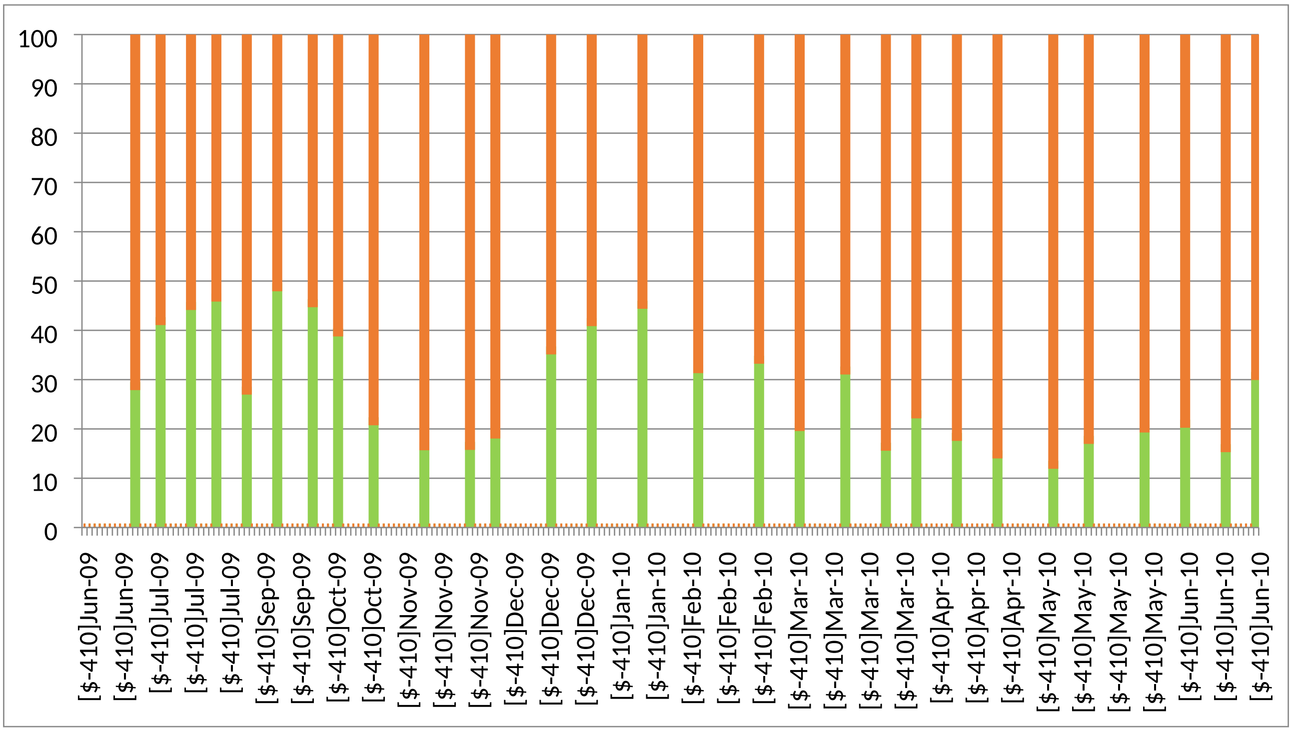
Supplementary material



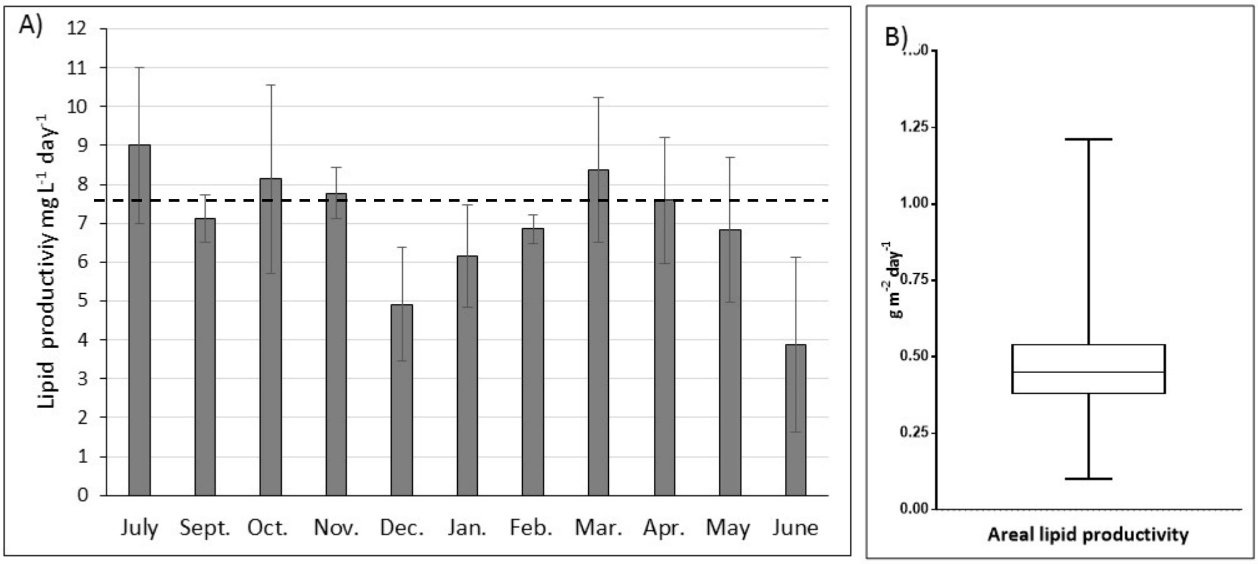
SM_Figure 1: Design of aeration system of the photobioreactor used in this study. Cultures were mixed-up by air sparging (5 L/ min) through three perforated pipe spargers running along the lower side of the culture chamber (hole size diameter: ca 10-15 µm) and a large diffuser tube (hole diameter: 33 mm) located at the bottom of the culture chamber.



SM_Figure 2. Cell concentration (expressed as cell ml^{-1}) along the fed-batch cultivation during one year experimentation . Cells were inoculated at ~10000 cell ml^{-1} and were cultivated under fed-batch conditions until they reached the first point of senescent phase.



SM_ Figure 3 : Distribution of saturated and unsaturated fatty acids along the annual experimentation. Orange: % unsaturated fatty acids; Green: % of saturated fatty acids



SM_Figure 4: (A) Monthly average lipid productivity, expressed as mg L^{-1} $day^{-1.}$ The dashed line represent the average value of annual lipid productivity (7.5 mg L^{-1} day^{-1}); (B) Boxplot of areal annual lipid productivity, expressed as g m^{-2} day^{-1}.

Dependent variable	Independent variable	*r*	r^2	*F*	*F sign*	*Equation*
Biomass productivity	Max Temperature	0.42	0.35	6.50	0.03	$y = -1.9746x + 107.96$
Biomass productivity	Min Temperature	0.57	0.33	4.37	0.07	$y = -1.92x + 80.48$
Biomass productivity	Temperature mean	0.61	0.38	5.45	0.04	$y = -1.9728x + 94.484$
Biomass productivity	Irradiance	0.08	0.01	0.06	0.81	$y = -0.2852x + 63.726$
Biomass productivity	T. mean and Irradiance	0.45	0.31	3.22	0.09	$y = -2.46x_1 + 1.02x_2 + 89.05$

SM_Table 1:

Linear regression analysis and multiple linear regression analysis between biomass productivity and Max Temeperature, Min Temperature, Temperature mean and irradiance.