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Development of a monitoring tool based on fluorescence and climatic data for pigments profile estimation in Dunaliella salina

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Abstract:	When growing microalgae for biorefinery processes, a high product yield is desired. For that reason, monitoring the concentration of the desired products during growth and products induction procedure is of great interest. 2D Fluorescence spectroscopy is a fingerprinting technique, used in situ and at real time, with a high potential for online monitoring of biological systems. In this work, D. salina pigments content were monitored using fluorescence data coupled with chemometric tools. Climatic parameters were also used as input variables due to their impact on the pigments profile in outdoor cultivations. Predictive models were developed for chlorophylls content (a, b and total) with variance captured between 50 and 90%, and R 2 varying between 0.6 and 0.9 for both training and validation data sets. Total

	carotenoids models captured 70% to 80% of variance, and R 2 between 0.7 and 0.9, for training and validation. Models for specific carotenoids (zeaxanthin, α -carotene, all-trans-β-carotene and 9-cis-β-carotene) captured variance between 60% and 90%, with validation and training R 2 between 0.6 and 0.9. With this methodology it was possible to calibrate a monitoring tool for pigments quantification, as a bulk and as individual compounds, proving that 2D fluorescence spectroscopy and climatic data combined with chemometric tools can be used to assess simultaneously and at real time different pigments in D. salina biomass production.
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Development of a monitoring tool based on fluorescence and climatic data for pigments profile estimation in *Dunaliella salina*

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Abstract

When growing microalgae for biorefinery processes, a high product yield is desired. For that reason, monitoring the concentration of the desired products during growth and products induction procedure is of great interest. 2D Fluorescence spectroscopy is a fingerprinting technique, used *in situ* and at real time, with a high potential for online monitoring of biological systems. In this work, *D. salina* pigments content were monitored using fluorescence data coupled with chemometric tools. Climatic parameters were also used as input variables due to their impact on the pigments profile in outdoor cultivations. Predictive models were developed for chlorophylls content (*a*, *b* and total) with variance captured between 50 and 90 %, and R^2 varying between 0.6 and 0.9 for both training and validation data sets. Total carotenoids models captured 70% to 80% of variance, and R^2 between 0.7 and 0.9, for training and validation. Models for specific carotenoids (zeaxanthin, α-carotene, all-trans-β-carotene and 9-cis-β-carotene) captured variance between 60% and 90%, with validation and training R^2 between 0.6 and 0.9. With this methodology it was possible to calibrate a monitoring tool for pigments quantification, as a bulk and as individual compounds, proving that 2D fluorescence spectroscopy and climatic data combined with chemometric tools can be used to assess simultaneously and at real time different pigments in *D. salina* biomass production.

Keywords: 2D fluorescence spectroscopy; EEMs (Excitation-Emission matrices); PLS modeling; Dunaliella salina; Carotenogenesis

1. Introduction

In the current industrialization of compounds from biological sources, such as microalgae-based biorefinery, the control and monitoring of the cultivation and process parameters rely on physical, chemical and biological analysis. Physical and chemical parameters are currently monitored online using sensors like pH, temperature, dissolved O_2 or CO_2 (Ulber et al. 2003; Glindkamp et al. 2009). However, biological parameters, such as cell concentration or product formation, are still mostly performed off line, where a sample has to be withdraw from the cultivation/process and subjected to different laborious and time-consuming procedures, losing the window of opportunity to take decisions at real time (Henriques et al. 2010). The development of an *in situ* and online sensor that could deliver a continuous stream of information would result in very short response time, enabling important control decisions in the spot. In fact, some studies were already reported intending to develop tools to monitor microalgae cultivation at real time

(Sandnes et al. 2006; Karakach et al. 2015; Sá et al. 2017; Nguyen and Rittmann 2018; Lai et al. 2019). The possibility of measuring several parameters simultaneously, without the need to sample, would be a great advantage for the microalgae biorefinery. Several spectroscopies have been studied for this purpose, namely fluorescence (Tartakovsky et al. 1996; Shaw et al. 1999; Schügerl 2001; Hantelmann et al. 2006; Glindkamp et al. 2009; Galinha et al. 2012; Ranzan et al. 2012; Biechele et al. 2015).

Fluorescence spectroscopy is able to measure several analytes simultaneously by scanning through a wide range of excitation/emission wavelengths (two dimensional (2D) scanning) (Tartakovsky et al. 1996; Lakowicz 2006; Biechele et al. 2015). This technique is non-invasive and highly sensitive to the presence of natural fluorophores, intra or extracellular. It is also reported that fluorescence spectroscopy can indirectly provide information about compounds that are not fluorophores but that interfere with the fluorophores in the sample (Ulber et al. 2003). Due to the complexity of fluorescence excitation-emission matrices (EEM), the use of chemometrics tools is advised to extract quantitative information from the fluorescence spectra and to resolve the occurrence of some limitations, such as inner filter effects or quenching, specially at high concentrations. The possibility of coupling an optical probe makes this technology suitable to be used in different processes within the biorefinery concept for an online and *in situ* monitoring (Galinha et al. 2011; Benito-Peña et al. 2016; Pawlowski et al. 2016; Sá et al. 2017).

In this study, the microalga *Dunaliella salina* was selected for its current industrial production of biomass retailed as natural source of carotenoids. Its ability to grow in hypersaline lagoons, with low nitrogen and high solar light intensity, protects the culture from contaminants and increases the carotenoid content, being β-carotene present at higher concentration (more than 12% of its dry weight) (Ben-Amotz 2004; Hu et al. 2018). Photosynthetic organisms, like *D. salina*, harvest light energy due to their pigments, which can be chlorophylls, carotenoids and/or phycobilins. Of those, chlorophyll and carotenoid molecules are known for being used as natural colorants and antioxidants in different food products (Schoefs 2002; Dufossé and de Echanove 2005; Saini and Keum 2019). Carotenoids are also important nutraceuticals, due to their anti-oxidant, anti-ageing, anti-inflammatory, anti-angiogenic, cardio and hepatoprotective properties (Hu et al. 2018).

The development of a monitoring tool able to detect all these pigments at real time will enable a better understanding of the pigments formation through the life cycle of *D. salina*, and at industrial scale it is useful to increase the biomass potential in a biorefinery context. Therefore, in this study, fluorescence EEMs were acquired during several pilot scale cultivation experiments of *D. salina*. Predictive models using Principal Component Analysis (PCA) and Projection to

Latent Structures (PLS) were developed in order to correlate the EEMs with the pigments profile using two different off-line calibration tools: spectrophotometric and HPLC methodologies. The importance of environmental conditions during the carotenogenesis and their impact on the pigments profile was also studied through the use of climatic parameters as input variables.

2. Material and Methods

2.1. *Dunaliella salina* growth and carotene induction conditions

D. salina DF40 was collected from Monzón Biotech (Spain), isolated by Marine Biological Association (United Kingdom) and scaled-up and produced at pilot scale by A4F - Algae for Future (Lisbon, Portugal).

Batch cultivation was carried out in artificial saltwater (ASW) under continuous aeration and mixed by bubbling using 0.2 μm-filtered atmospheric air enriched with 2% of CO_2. The temperature was set at 25ºC and continuous illumination was provided by fluorescent tubes (150 µmol m^{-2}.s).

For carotenoid induction experiments, the culture was scaled-up to pilot scale flat-panel photobioreactors (Green Wall™, GW) and the same ASW medium was used with depletion of nitrogen and increased salinity. All batches were performed between January and October of 2017, exposed to outdoor weather conditions, with control of maximum temperature, but no control of the minimum temperature. In total, six batches were performed and monitored from the inoculation, with non-stressed "green" cells, until reaching a highly stressed "orange" culture.

2.2. Sampling procedure and analysis

Samples were taken every other day until a stable chlorophylls/carotenoid's ratio was reached. For each sample, pigments quantification and fluorescence spectroscopy assessment were performed.

2.2.1. Pigments analysis

Pigment quantification was performed by two methodologies, spectrophotometry and HPLC.

Briefly, *D. salina* cells (2 mL) were collected by centrifugation (5000 g, 5 min) and resuspended with 2 mL of 100% methanol. Samples were sonicated for 5 min and then incubated at 60ºC for 40 min, followed by cooling on ice for 15 min. After centrifugation, the supernatant was collected, and the extraction was repeated until a white pellet was achieved. Quantification was performed in a UV/Vis spectrophotometer (Leu and Hsu 2005). Modified's Arnon's equations were used to calculate chlorophyll and carotenoid contents (Liechtenthaler 1987):

$$Chl_a = (16.72 \times A_{665} - 9.16 \times A_{652}) \times dilution\ factor\ [\text{mg L}^{-1}]$$

$$Chl_b = (34.9 \times A_{652} - 15.28 \times A_{665}) \times dilution\ factor\ [\text{mg L}^{-1}]$$

$$Chl_{tot} = Chl_a + Chl_b\ [\text{mg L}^{-1}]$$

$$Car_{tot} = (dilution\ factor \times 1000 \times A_{470} - 1.63 \times Chl_a - 104.96 \times Chl_b) / 221\ [\text{mg L}^{-1}]$$

Two different methods were used to quantify pigments by HPLC, one for chlorophylls and another for carotenes. The HPLC system consisted of a Waters Alliance Separations Module e2695 (Waters, Dublin, Ireland) coupled to a Photodiode Array Detector Module e2998 (HPLC-PDA).

For chlorophyll analysis, the extraction was performed in fresh biomass pellet using 100% methanol at 4 ºC, repeatedly until a white pellet was reached. Separation of chlorophyll *a* and *b* was achieved using a reverse phase C18 (Vydac 201TP^{TM}, 250 x 4.6 mm, 5 µm, Hichrom, Berkshire, United Kingdom) and an isocratic elution with 100% methanol, at a constant flow rate of 1 mL min^{-1}. The column oven temperature was set at 25 ºC and the injection volume was 20 µL.

For carotene analysis, the extraction was performed in fresh biomass pellet with 100% methyl tert-butyl ether (MTBE). After centrifugation, the supernatant was recovered, and the extraction was repeated until a white pellet was obtained. Separation of carotenes was achieved using a reverse phase C30 (YMC Carotenoid, 250 x 4.6 mm, 5 µm, YMC Europe GmbH, Dislaken, Germany) and an isocratic elution with 90% methanol and 10% MTBE, at a constant flow rate of 1 mg L^{-1}. The column oven temperature was set at 25ºC and the injection volume was 20 µL.

Standard stock solutions of chlorophylls (0.1 mg L^{-1}) and carotenes (0.2 mg L^{-1} for all-trans-β-carotene, 9-cis-β-carotene and α-carotene; 0.1 mg L^{-1} for lutein and zeaxanthin) were prepared with 100% methanol and 100% MTBE, respectively. For each compound, standards were diluted from the stock solutions to obtain a concentration range between 0.0025 mg L^{-1} and 0.1 mg L^{-1}. Independent replicates were prepared from the stock solutions in the beginning of each run and were used to assess the precision of the method. All stock solutions and calibration standards were stored at 4 ºC.

2.2.2. 2D Fluorescence spectroscopy

All culture samples collected for pigments analysis were also analyzed by 2D fluorescence spectroscopy directly through the immersion of an optical fiber probe in a stirred sampling tube, preventing cell sedimentation. Fluorescence EEMs were acquired using a fluorescence spectrophotometer Varian Cary Eclipse, equipped with excitation and

emission monochromators, and with a fluorescence optical fiber probe. As described in previous work (Sá et al. 2017), fluorescence data was collected in an excitation wavelength range of 250 to 690 nm, with an excitation slit of 10 nm and increments of 5 nm, and emission wavelength between 260 and 700 nm, with an excitation slit of 20 nm and increments of 5 nm.

2.3. Climatic data

Carotenoid induction experiments were performed in A4F - Algae 4 Future outdoor facilities, thus climatic conditions at which the experiments were exposed were used as input in the development of PLS models. Two strategies were attempted using the climatic data: i) based on the values assessed on the sampling day; ii) using cumulative approach, where the values used for each parameter are the sum of the values assessed from time zero until the sampling day. The climatic parameters used are shown in Table 1.

2.3.1. Temperature range, precipitation and sunlight

Temperature (ºC) and precipitation (mm) data were taken from the ERA Interim global atmospheric reanalysis produced by the European Centre for Medium-Range Weather Forecasts (ECMWF) for the region of Lisbon (Dee et al. 2011). Maximum and minimum temperature products, taken at 2 m from the surface at every 6 hours, were used to calculate the temperature range. The daily total precipitation input was the sum between the convective and stratiform precipitation, and used as accumulated precipitation of the day. Sunlight data (in minutes) was obtained from the website timeanddate.com, with measurement location at Lisbon Portela Airport, 3.8 km from A4F experimental unit. Through the hour of sunrise and sunset was determine the day length in hours per day.

2.3.2. FPAR and clouds fraction

The fraction of photosynthetically active radiation (FPAR) is defined as the fraction between the wavelengths 400 and 700 nm of incident photosynthetically active radiation that is absorbed by the green elements of vegetation. This parameter is important when measuring biomass production because vegetation development is related to the rate at which radiant energy is absorbed. FPAR data was acquired by the MCD15A3H version 6 MODIS (Moderate Resolution Imaging Spectroradiometer) Level 4, as a 4-day composite data set with a 0.5-km-pixel resolution for the Lisbon area (Myneni et al. 2015).

Cloud fraction is defined by the Earth fraction which is covered by clouds relative to the fraction that is not covered. Clouds play an important role in regulating the amount of energy that reaches the Earth from the Sun and also the

158 energy that the Earth reflects back into space. This parameter is a product of radiance and reflectance measurements
159 acquire by Cloud Mask (MOD 35) of MODIS, as a daily data set with a 1-km-pixel resolution. The data set was
160 acquired through NEO website (NASA Earth Observations).

161 **2.3.3. Irradiance**

162 Irradiance was recorded by WatchDog® Weather Station (Spectrum Technologies, Inc., Illinois, USA) at A4F-Algae
163 4 Future outdoor facilities, every 15 minutes. The value used in the PLS modeling corresponds to the mean value of
164 the day.

165 **2.4. Development of multivariate models**

166 The modelling methodology followed in this work is represented in Figure 1.

167 Firstly, Principal Component Analysis (PCA) was used to compress and deconvolute the fluorescence excitation-
168 emission matrices (EEMs) into principal components (PCs), by extracting the most relevant information. Shortly, the
169 first PC was selected for explaining the higher variance possible; then a second PC was selected for explaining the
170 higher remaining variance possible with the constrain of being orthogonal to the first one; and so on. The calculated
171 PCs are uncorrelated and ordered according to the variance explained. PCA was applied to all spectra acquired and
172 the first ten PCs were selected to be used as input in the projection to latent structure (PLS) modeling, since they
173 captured more than 99% of the variance.

174 Projection to Latent Structures (PLS) modeling was used to stablish multilinear correlations between the PCs of
175 fluorescence and climatic conditions (inputs) and the pigments concentration, chlorophylls and carotenoids (outputs).
176 The models were developed using 41 fluorescence spectra, corresponding to 41 samples. From this initial data set, 75
177 % was randomly select to train the model, i.e., to find the best model that explains the higher variability found in the
178 data with the lower errors. Then, the remaining 25% of the data were used to validate the model developed previously
179 and accessed the prediction quality. All data were normalized before being used in the PLS models.

180 For each pigment, three strategies were attempted based on the initial inputs used: first using only ten PCs from the
181 fluorescence; second, using PCs from the fluorescence and climatic data of the day of the sampling; third, using PCs
182 from the fluorescence and climatic data with accumulative effect towards the sampling day, which means, for each
183 sampling day was considered not only the values of that day, but also the values of the previous sampling days (one
184 cumulative parameter is the sum of all measures of that parameter until the day of sampling).

The selection of the useful PCs and climatic inputs for the models was performed by iterative stepwise elimination (ISE)(Boggia et al. 1997). The quality of each model was evaluated by the percentage of the variance captured, the slope and coefficients of determination (R^2) of the validation and the training data sets, as well as the root mean square errors of cross validation (RMSECV) and prediction (RMSEP). All algorithms were implemented in Matlab, using the n-way tool box for PCA and PLS through PARAFAC and nPLS functions, respectively (Andersson and Bro 2000).

3. Results and Discussion

The high contents of carotenoids that *D. salina* can accumulate is the reason why this microalga biomass is successfully industrialized and commercialized. To be able to develop a tool that can monitor the pigments profile in *D. salina* will increase the knowledge of the biological status enabling the operator to take relevant decisions at real time, like the optimal harvesting point. For that reason, six pilot-scale flat-panel experiments were performed to induce the increase of carotenoids in *D. salina*, and two main approaches were evaluated, the importance of using climatic inputs and the importance of choosing the adequate methodology to calibrate the models. Fluorescence EEMs were obtained for each sample and the pigments profile was quantified. The content of two chlorophylls (*a* and *b)* and five carotenes (lutein, zeaxanthin, α-carotene, β-carotene and 9-cis-β-carotene) were measured using two different methodologies: spectrophotometric and by HPLC.

Figure 2 shows the fluorescence spectra acquired from *D. salina* culture during carotenogenesis in the sixth batch for days one, six and fourteen after the inoculation. It is possible to distinguish 2 fluorescence regions, one at excitation/emission wavelengths of 275 nm and 300 to 350 nm, and another at higher emission wavelengths, above 650 nm. The first region is defined as protein-like fluorescence, mainly because of aromatic amino acid tryptophan fluorescence (Galinha et al. 2011). The second region is defined as the pigment band (Moberg et al. 2001). The differences between the spectra from the inoculation day (day one, Fig. 2a) and six days after (Fig. 2b) is quite visible, mainly due to the pigments band fluorescence intensity. In these samples the ratio carotenoid/chlorophyll increased from 0.21, on day one, to 3.04, on day six. However, when analyzing the fluorescence spectra of day fourteen (Fig. 2c) some differences can be also spotted, although the ratio carotenoid/chlorophyll was 3.01, similar to day six. During carotenogenesis experiments, the biomass concentration also increased through time, resulting in the intensification of the emission and excitation light scatter. It is noteworthy that fluorescence was assessed directly on the samples, without any dilution or sample preparation, as it would be if it was measured using the optical probe directly coupled

to the bioreactor. As previously described, in stress conditions, the cellular growth rates are reduced and cells become larger, with the optical properties of cell suspensions changing due to size and shape of the cells interacting with radiation (Merzlyak et al. 2007; Farhat et al. 2011). Furthermore, the direct quantitative analysis of pigmented cell suspensions through optical methods is hampered by scattering (Merzlyak et al. 2007). Therefore, to use fluorescence as a monitoring tool, the effect of increased turbidity and color must be seen as a source of information, thus, chemometric tools are needed to extract quantitative information.

Three input strategies were studied to develop the PLS models for prediction of each compound individually: without climatic inputs; and with daily or cumulative climatic inputs. However, all models needed the input of the climatic parameters to successfully estimate the pigments content, which validates the importance of climatic conditions on microalgae cultivation in outdoors pilot facilities. Therefore, the models developed without climatic conditions inputs are not shown in this work. The use of daily data as input can give extra information about the impact of climatic conditions on the culture status of the sampling day, although the cumulative effect can be important when studying the induction of carotenes.

The most relevant models achieved for chlorophylls and carotenoids are shown in Table 2 and 3, respectively.

3.1.Chlorophylls

3.1.1. Chlorophyll *a*

For Chlorophyll *a* modelling, and using spectrophotometric methodology as calibration method, two models are presented, one with daily climatic inputs and another with cumulative climatic inputs. In this case, the use of cumulative climatic inputs did not improve greatly the quality of the model, although a slight increase was observed. The variance explained increased from 83.3 %, for daily climatic inputs, to 89.2 %, for cumulative. An important difference between the models is the selection of the inputs used by each model. When calibrating the model with daily climatic inputs, less inputs were selected in general, either from fluorescence PCs or climatic. Only one climatic input was selected, the fraction of photosynthetically active radiation (FPAR), an important parameter in this study, and particularly for this model, because of the direct correlation between photosynthetic activity and content of green pigments. When calibrating with cumulative climatic data, more fluorescence PCs were selected, as well as two different climatic inputs, the presence of clouds and irradiance. The irradiance is a parameter that indicates the amount of light that reaches the Earth surface and was measured daily at the A4F - Algae for Future facilities. Unlike FPAR

measurements, all light flux is considered in irradiance measurements. The presence of clouds can also give an indication of the amount of light that is being blocked from the Earth surface. Although different light measurements are used to explain the variability observed in these experiments, it is safe to enhance the importance of light settings when working in outdoor systems such as in this study.

Using a different methodology to calibrate the model, like HPLC, several differences are noteworthy, such as less fluorescence PCs inputs were selected. However, less variance was explained (69.7 %) and the prediction error (RMSEP) was higher. This tendency was also observed in the experimental error of the methodologies, 3.99×10^{-10} mg cell^{-1} for HPLC and 1.16×10^{-10} mg cell^{-1} for spectrophotometer, which can be also related with differences between extraction methodologies (sonication and 60ºC were used for the spectrophotometric analysis, and 4ºC for HPLC analysis). However, the average chlorophyll *a* concentration was higher when measured with HPLC methodology, thus, the error related to the average for both methods was similar (around 5%).

Different methods for pigments quantification and extraction have been discussed in the literature for the past years. For different microalgae or biological state ("green" or "orange"), the selection of the extraction methodology and the analytical method should be carefully chosen. In this study, when aiming to monitor chlorophyll *a* in *D. salina*, similar models were achieved using HPLC or spectroscopic data. However, the spectrophotometer method presented lower error values being a more accurate method. Since this methodology is less laborious and less time consuming, using it for the model calibration can be an advantage.

3.1.2. Chlorophyll *b*

When aiming to monitor chlorophyll *b*, differences in the methodology to calibrate the model are also noticeable. Although daily climatic inputs were provided in the model calibrated with spectrophotometer quantification, none of the parameters were selected. Together with a low number of fluorescence PCs selected by the model, a low variance was explained (47.5 %). When using HPLC to calibrate the model, a higher R^2 for both training and validation sets was observed. However, the difference between RMSEP and RMSCV shows that the model is not robust to predict chlorophyll *b* content. Furthermore, high RMSECV may indicate some degree of overfitting (most probably due to the high number of inputs required). Like the tendency observed for chlorophyll *a*, chlorophyll *b* concentration models have a higher RMSEP when using HPLC results than spectrophotometric. However, this tendency was not observed in the analytical error of the methodologies, 7.73×10^{-11} mg cell^{-1} for HPLC and 1.52×10^{-10} mg cell^{-1} for spectrophotometry.

The RMSEP values for monitoring chlorophyll *b* content were higher than desired. The high standard deviation found on the analytical measurements interferes greatly with the model calibration, leading to strong limitations. These findings confirm the importance of carefully choosing the analytical methodology for both extraction and quantification. Furthermore, monitoring chlorophyll *b* content by 2D fluorescence spectroscopy could be enhanced with the addition of more experimental points through a longer calibration time. Nevertheless, even with the low accuracy due to the results of the analytical method for the model calibration, the possibility of using this technology online is a great advantage since none of the current methodologies enables it.

3.1.3. Total chlorophylls

After analyzing the individual content of each chlorophyll, and due to the high variability observed, a different approach was attempted to monitor the chlorophyll content as total amount, by the sum of chlorophyll *a* and *b* peak areas obtained with HPLC analysis. A variance of 74.2 % was explained using nine of the ten PCs of fluorescence and several cumulative climatic parameters. Similar R^2 for validation and training data sets (0.79 and 0.74, respectively) and a small difference between RMSEP and RMSECV were observed.

When comparing all the models for chlorophyll *a*, *b* and total content, less climatic inputs are needed to predict chlorophyll *a* content, with more variability explained. Regarding the two analytical methodologies, spectrophotometric and HPLC analysis, due to a different extraction procedure, it is difficult to assert which one is more suitable. Slightly better results were obtained for models calibrated with spectrophotometric analysis, but that can be related with the extraction procedure. To confirm this approach, the impact of the heat versus room temperature extraction using the same apparatus should be further tested.

Through time, simultaneous quantification of chlorophylls and the interferences between them has been a topic of study by several authors. Moberg et al. (Moberg et al. 2001) used 2D fluorescence spectroscopy to analyze a mixture of six standard pigments: chlorophylls *a*, *b* and *c*, and their respective degradation products, pheophytins *a*, *b* and *c*. The authors observed that the signals from the different pigments overlap, and for that reason, their direct quantification using only a specific region of the spectra can be misleading. When validating 2D fluorescence spectroscopy using HPLC they found that the RMSEP for chlorophyll *a* was six times higher than with fluorescence spectroscopy. Also, chlorophyll *b* quantification was not always possible due to limitations in the quantification limit of the HPLC methodology (Moberg et al. 2001). Fluorescence spectroscopy was also previously applied for online estimation of biomass and proteins during cultivation of microalgae *Scenedesmus* (Karakach et al. 2015). However,

that application required a constant intracellular chlorophyll content to enable the estimation of biomass concentration (Karakach et al. 2015). Yet, the present work shows that by using a broader excitation wavelength range and climatic data (accounting for operating conditions) it is also possible to estimate the content of chlorophylls (*a*, *b* or total) in a highly variable range of concentrations during induction of carotenes in *D. salina*.

The models presented were selected for their better results on the prediction of chlorophylls content (*a*, *b* or total) when using 2D fluorescence spectroscopy as input. It is noteworthy that most of these models also used climatic data as input, either as daily or cumulative. To our knowledge, this approach of using climatic inputs to help monitor and predict pigment induction online was not used before. The results obtained enhance the importance of using the operating conditions (climatic data), coupled with the 2D fluorescence spectroscopy, to monitor chlorophylls profile when cultivating *D. salina* in outdoor facilities.

3.2. Carotenoids

3.2.1. Total carotenoids

When aiming to predict total carotenoids content in *D. salina* biomass, better result was achieved when using spectrophotometric quantification to calibrate the model, and also using cumulative effect of the climatic data. Lower RMSEP (2.51×10^{-9} mg cell^{-1}) and a lower RMSECV (3.85×10^{-9} mg cell^{-1}) were observed, indicating the robustness of the model, and higher R^2 for validation and training data sets (0.89 and 0.82, respectively). For both models, all climatic inputs were selected, once again revealing the importance of the climatic conditions in the outdoor cultivation of microalgae.

It is well-known that there is no harmonized protocol for carotenoids extraction, with many options available in literature depending on physical characteristics, composition and amount of water in the samples, although a chromatographic separation seems to be unanimous. Nevertheless, optimization of the chromatographic separation is required to obtain a good resolution of peaks, which is not always achieved, due to the complexity of these extracts and presence of compounds with similar structures. Petri et al. study on orange fruit extract showed that, when analyzing 52 carotenoids by LC-DAD-MS/MS, most of them co-elute, revealing the challenge of separating and quantifying every single carotenoid (Petry and Mercadante 2016). Through the HPLC methodology used it was possible to discriminate several different pigments like zeaxanthin, *α*-carotene, all-*trans*-*β*-carotene and 9-*cis*-*β*-

carotene. Therefore, different models were developed to assess each compound individually (as shown in Table 3 and in Figure 3).

3.2.2. Zeaxanthin

Zeaxanthin is widely present in photosynthetic multicellular organisms, including microalgae, and is always found together with lutein and *β*-carotene (Zhang et al. 2018). Two models were selected to monitor zeaxanthin content in *D. salina* cells, using daily and cumulative data. For both models, two fluorescence PCs were selected, and the number of hours of sunlight was a common climatic input. However, the use of cumulative climatic data and the inclusion of the irradiance information, led to a model able to explain more data variability (79.3%), with a slight increase on RMSEP. It is noteworthy the low number of inputs required to predict zeaxanthin, and the similarity between RMSEP and RMSECV, meaning that robust models were achieved based on fluorescence and sunlight.

3.2.3. α-Carotene

The model developed has the highest variance explained of all carotenoids in this study (87.8 %). The climatic parameters with impact for the determination of *α*-carotene were the thermal amplitude, precipitation and parameters related with light, such as number of hours of sunlight, FPAR and irradiance. When studying the influence of cumulative effect of climatic parameters, the models obtained did not revealed an improvement (data not show).

3.2.4. β-Carotene

β-Carotene is the carotenoid present in higher concentration in *D. salina*. Analysis of the oily globules' structures where *β*-carotene is stored showed mainly two stereoisomers: all-trans and 9-*cis*. It is reported in the literature that the light intensity at which the cultures are exposed influences greatly the final ratio between all-trans and 9-*cis*, and also in the total amount of *β*-carotene accumulated within each cell (Ben-Amotz 2004).

For both forms of *β*-carotene, the models where climatic parameters were used as cumulative effect led to higher variance explained when compared with the ones when using daily climatic inputs. For all-trans-*β*-carotene, the use of cumulative climatic inputs also decreased both errors, RMSEP and RMSECV. Between the climatic parameters provided, the exposure to sun light was important to explain the development of carotenoids during nitrogen starvation, especially when referring to pilot-scale outdoor *D. salina* cultivation. For 9-cis-β-carotene, slightly higher errors (RMSEP and RMSECV) were observed when using the climatic input as cumulative, however the difference between them was lower, revealing a more robust prediction model.

In general, all four carotenes models presented similar results. When plotting the models (Figure 3) it is possible to observe some dispersion of the values, resulting in lower R^2, but no outliers were found, which lead us to conclude that performing more assays would benefit the prediction capability. This study proves that 2D fluorescence spectroscopy is a powerful tool that can simultaneously detect and quantify different carotenoids, with the advantageous of giving an estimation at real time.

3.3. Application perspectives

It is known today that, for the microalgae industry to be as competitive as other plant-based refineries, the biorefinery concept needs to be improved and the tendency is to produce and recover high value compounds. The industrialization of microalgae increased the need for better methodologies to monitor (and control) these complex biological systems. Among several advantages, 2D fluorescence spectroscopy can be coupled with an optical probe directly immersed in the bioreactor/system under study, and enables the collection of information frequently and at real time, without the need for sampling.

When aiming for pigment content monitoring, there are several methodologies in the literature, but they require a sampling step and extraction procedure, which normally are time consuming analysis. Other spectroscopic and fluorometric methods studied so far also require sampling, either for extraction of pigments, for dilution or for the use of other methods able to remove interferences that prevent a direct correlation (Kleinegris et al. 2010; Solovchenko et al. 2013; Chen et al. 2017). Furthermore, such tests aim at measuring total pigments or a specific compound but are not able to discriminate simultaneously between different pigments. Therefore, even if the accuracy of the prediction model can be lower than the analytical quantification, the possibility of having several and repeated fluorescence measurements at real time is a motivating advantage in industrial production.

For an industrial application, the prediction accuracy of such models as developed in the present work, can be improved with the increase of the calibration period, allowing the collection of more data points to attenuate the prediction error and to account for higher variability of the operating conditions. Also, the incorporation of extra information about the cultivation process, such as parameters that are known to influence the final biomass composition (like media composition) can possibly lead to an improve in the model's prediction capability.

4. Conclusions

The approach presented in this work validates the use of 2D fluorescence spectroscopy and climatic data combined, with the use of chemometric tools, as monitoring tool for pigments as a bulk, total chlorophylls and total carotenoids, but also as individual compounds, in *D. salina* biomass.

Mathematical models were successfully developed to assess chlorophylls content as chlorophyll *a*, *b* and total chlorophyll, and carotenoids content as total content, and specifically, as zeaxanthin, α-carotene, all-trans-β-carotene and 9-cis-β-carotene. To validate the models, a careful choice of the analytical method is required, since experimental errors and accuracy of the analytical methods will highly influence the model prediction ability. Furthermore, the possibility to include climatic data, related with the environmental conditions during microalgae production in outdoor facilities, enhances the prediction of pigments content. Additionally, the possibility of using a fluorescence probe directly in the production system avoids the need of sampling and of an extraction step.

Although the analytical methodologies commonly used (such as HPLC) are accurate and able to assess different compounds simultaneously, they are expensive, time consuming and require a laborious extraction step, while 2D fluorescence spectroscopy (coupled with appropriate mathematical tools) proved to be an effective solution for the industrial application to address the monitoring of these high value and in high demand compounds.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Table 1 Climatic parameters used in the development of PLS models

INPUT #		UNITS	DATA SOURCE	FREQUENCY
11	Temperature	ºC	ECWMF	daily
12	Total Precipitation	mm	ECWMF	daily
13	Sunlight	min	timeanddate.com	daily
14	Cloud	dimensionless	MODIS/MOD06	daily
15	Fpar	dimensionless	MODIS/MCD15A3H.006	4-days
16	Irradiance	$MJ/m^2.day$	measured locally	daily

Table 2 Statistical parameters of the selected models for chlorophylls prediction. The climatic inputs code is shown in Table 1

	Analytical Method	*Type of inputs*	*Var (%)*	*RMSEP (mg cell^{-1})**	*RMSECV (mg cell^{-1})**	*Validation*		*Training*		*Selected Inputs*	
						R^2	Slope	R^2	Slope	Fluorescence PCs	Climatic
Chl a	*Spect*	daily	83.3	$4.42x10^{-10}$	$7.17x10^{-10}$	0.85	0.99	0.83	1.00	1 2 4 5 6 8 9 10	15
		cumulative	89.2	$5.97x10^{-10}$	$6.26x10^{-10}$	0.78	0.85	0.89	1.01	1 2 3 4 5 6 7 8 9 10	14 16
	HPLC	cumulative	69.7	$2.04x10^{-09}$	$3.17x10^{-09}$	0.71	1.25	0.70	1.00	1 2 3 6 8 10	15 16
Chl b	*Spect*	daily	47.5	$2.99x10^{-10}$	$5.76x10^{-10}$	0.75	1.17	0.47	1.00	3 4 5 6 9 10	-
	HPLC	cumulative	67.8	$3.77x10^{-10}$	$9.27x10^{-10}$	0.79	0.86	0.68	1.00	1 2 3 4 5 6 7 8 9 10	12 14 15 16
Total Chl	*HPLC*	cumulative	74.2	$8.20x10^{+04}$	$1.25x10^{+05}$	0.79	0.88	0.74	1.00	1 2 4 5 6 7 8 9 10	11 12 13 14 16

Spect – Spectrophotometric; Chl *a* – Chlorophyll a; Chl *b* – Chlorophyll b; Total Chl – Total Chlorophyll.

(*) RMSEP and RMSECV values are presented as mg cell^{-1}, except for the models of total Chlorophylls, which are in area units.

Table 3 Statistical parameters of the selected models for carotenoids prediction. The climatic inputs code is shown in Table 1

	Analytical Method	*Type of inputs*	*Var (%)*	*RMSEP (mg cell^{-1})**	*RMSECV (mg cell^{-1})**	*Validation*		*Training*		*Selected Inputs*	
						R^2	Slope	R^2	Slope	Fluorescence PCs	Climatic
Total Carotenoids	*Spect*	daily	79.6	$2.75x10^{-09}$	$4.54x10^{-09}$	0.79	1.04	0.80	1.00	1 2 3 4 5 6 7 8 9 10	11 12 13 14 15 16
		cumulative	81.7	$2.51x10^{-09}$	$3.85x10^{-09}$	0.89	0.96	0.82	1.00	1 2 3 4 6 7 8 9 10	11 12 13 14 15 16
	*HPLC**	cumulative	64.9	$6.30x10^{+06}$	$7.35x10^{+06}$	0.72	0.91	0.65	1.00	2 3 7	12
Zeaxanthin		daily	74.4	$1.94x10^{-10}$	$1.99x10^{-10}$	0.69	0.86	0.74	1.00	2 3	13
		cumulative◄	79.3	$2.52x10^{-10}$	$1.78x10^{-10}$	0.62	1.11	0.79	1.01	3 8	13 16
α-Carotene		daily◄	87.8	$4.90x10^{-10}$	$3.21x10^{-10}$	0.63	1.27	0.88	1.00	1 2 3 4 5 6 7 8 10	11 12 13 15 16
all-trans-β-Carotene		daily	70.4	$4.97x10^{-09}$	$4.61x10^{-09}$	0.79	1.47	0.70	0.99	1 2 3 6 8	12 13
		cumulative◄	79.1	$3.31x10^{-09}$	$4.05x10^{-09}$	0.80	1.18	0.79	1.00	2 3 4 10	13 16
9-cis-β-Carotene		daily	64.2	$2.17x10^{-09}$	$3.80x10^{-09}$	0.73	0.83	0.64	1.00	2 3 6	13
		cumulative◄	65.7	$2.65x10^{-09}$	$3.73x10^{-09}$	0.88	1.29	0.66	1.00	2 3 4 5 6 7 8 10	11 12 13 14 15 16

Spect – Spectrophotometric;

(*) RMSEP and RMSECV values are presented as mg cell^{-1}, except for the models of total carotenes by HPLC, in area units.

(◄) Models represented in Fig. 3.

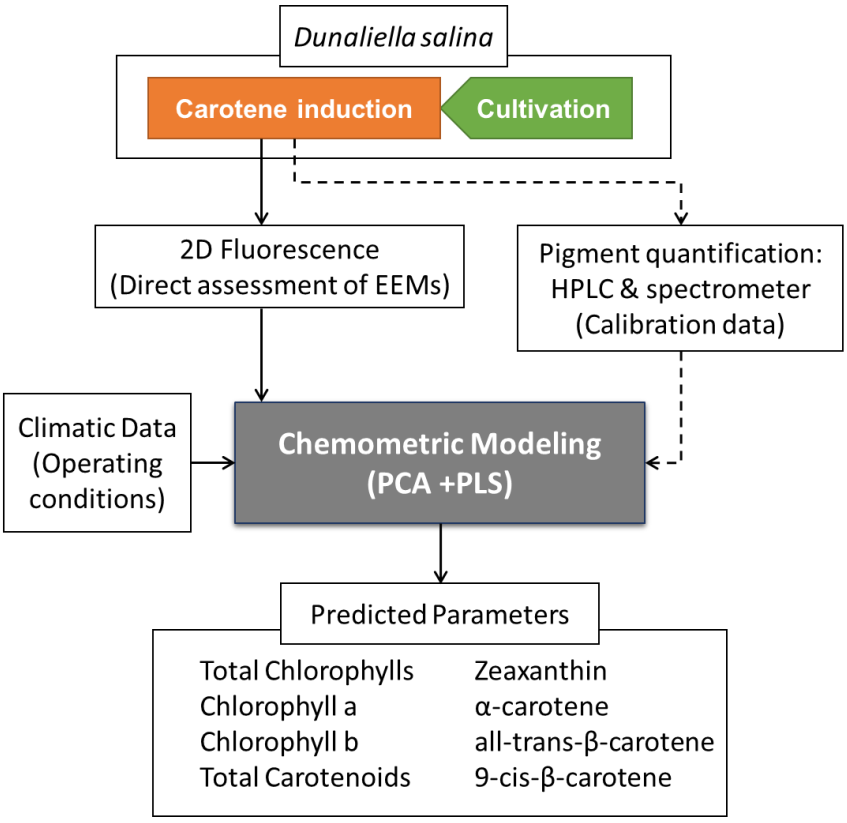

Fig. 1 Schematic representation of the methodology followed. Dashed arrows represent the offline measurements required for model calibration

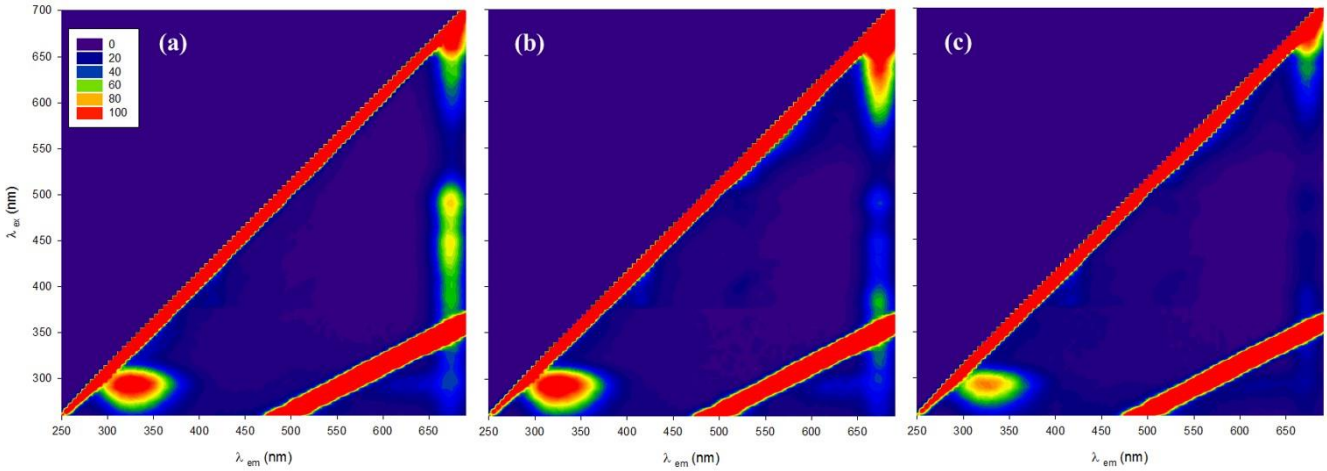

Fig. 2 2D Fluorescence spectra of *D. salina* during one carotenogenesis batch; (a) day one (inoculation), (b) after six days, and (c) after fourteen days. X-axis displays the wavelengths of emission, Y-axis the wavelengths of excitation and the intensity of the fluorescence is represented through color gradient. Two distinct fluorescence regions can be noticed, a protein-like region (excitation wavelength of 275 nm and emission wavelengths between 300 and 350 nm) and a pigment band (emission wavelengths above 650 nm)

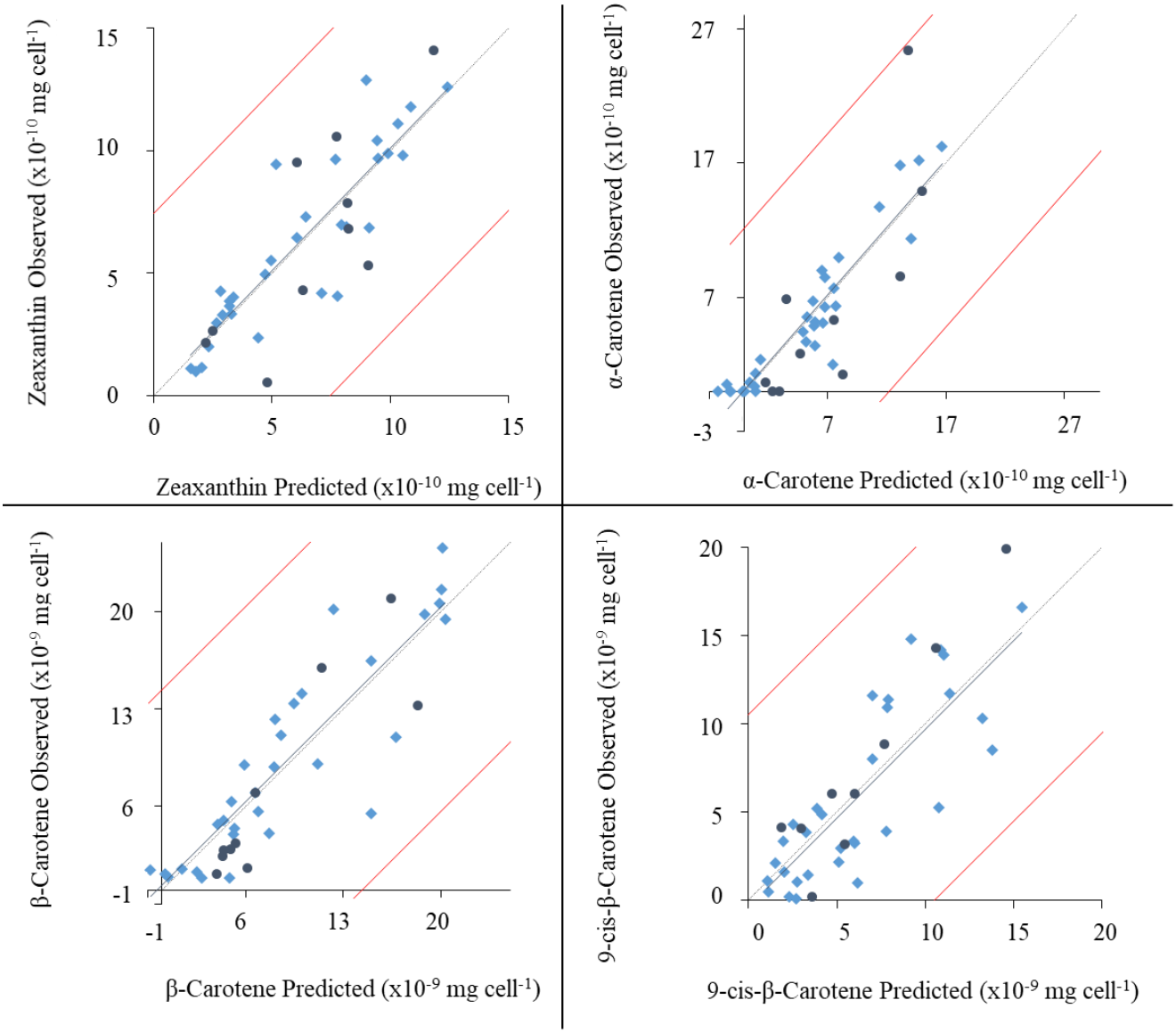

Fig. 3 Carotenoids concentration prediction models, from left to right and top to bottom: zeaxanthin, α-carotene, β-carotene and 9-cis-β-carotene. Training (♦) and validation (●) data are presented as mg cell^{-1}. Statistical parameters of the models represented are displayed in Table 3