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Perspectives of fluorescence spectroscopy for online monitoring in microalgae industry

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Summary

Microalgae industrial production is viewed as a solution for alternative production of nutraceuticals, cosmetics, biofertilizers, and biopolymers. Throughout the years, several technological advances have been implemented, increasing the competitiveness of microalgae industry. However, online monitoring and real-time process control of a microalgae production factory still require further development. In this mini-review, non-destructive tools for online monitoring of cellular agriculture applications are described. Still, the focus is on the use of fluorescence spectroscopy to monitor several parameters (cell concentration, pigments, and lipids) in the microalgae industry. The development presented makes it the most promising solution for monitoring up-and downstream processes, different biological parameters simultaneously, and different microalgae species. The improvements needed for industrial application of this technology are also discussed.

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Challenges of monitoring cell cultivations

Due to the increased interest for greener and renewable bio-based economies, algal biomass is being valorized for the sustainable production of food and feed, chemicals, fuels, and materials. Through the years, several technological advancements have been studied and implemented, increasing the competitiveness of biomass production. The introduction of closed cultivation systems, such as bioreactors, enabled the production under defined and controlled conditions, leading to optimized viability, reproducibility, and higher productivities. Following this trend for more competitive production systems, the need for online monitoring has emerged.

Within the biotechnological processes involved in a cell-based production, three types of parameters need to be monitored: physical, such as temperature and conductivity; chemical, such as pH, O_2 and CO_2 partial pressure (pO_2 and pCO_2); and biological, such as cell concentration and viability, substrate and product' concentrations. It is important to take into consideration that interactions between these three classes of parameters can occur and that these interactions are usually complex. Also, the parameters measured online are usually less than the required parameters needed for accurate monitoring and control (Ulber *et al.*, 2003; Glindkamp *et al.*, 2009). Currently, most of the monitoring is based on offline analysis of samples withdrawn from the cultivation or biorefinery processes. The sampling frequency is conservative to neither disturb the system nor contaminate it, and the metabolic activities must be stopped to show the status of the culture at a specific time. In addition, some results depend on time-consuming analytical procedures, making this sampling strategy not appropriate for direct process control (Henriques *et al.*, 2010).

Therefore, online monitoring and closer process control of bioreactors is a basic requirement for the development of efficient biological processes (Marose *et al.*, 1999; Schügerl, 2001).

The general requirements are usually selectivity, sensitivity, and response time (Dorresteijn, 1997; Olsson and

Nielsen, 1997; Glindkamp *et al*., 2009). Four configurations have been explored so far (Fig. 1): inline, where the medium is monitored directly; online, when the sensor is moved to a particular part of the bioreactor, like a bypass, so the bubbles from aeration do not interfere (for example); at-line, where a sample is withdrawn from the bioreactor but rapidly analyzed; and offline, when the sample is removed from the bioreactor, and there is a need for laborious laboratory work. With in-/online sensors, it is possible to acquire a continuous stream of information. Thus, biological systems can be monitored faster and more efficiently allowing an immediate response, leading to improved production processes with high-quality process control.

Nowadays, the most common in- and online sensors used in biotechnology are based on electrochemical principles such as pH, pO_2, and conductivity, and are well-known in the field. However, the need for efficient tools to monitor simultaneously a wide range of substrates and products and to control the cultivation environment increased the urgency for better solutions, and the use of optical sensors for online monitoring is emerging.

Spectroscopic optical sensors

With the improvement of optical fibre technology, efficient at larger distances and longer communication ranges, the optical sensor technology became very promising for in-/online monitoring. The principle of spectroscopic optical sensors is based on the interaction between light waves (absorption or luminescence) with the molecules. This technique presents several advantages for in-/online monitoring of complex biological systems, for example, industrial microalgae production and refinery. These sensors are non-invasive, non-destructive, and allow the detection of several molecules simultaneously. The need for sampling the system is low or inexistent, decreasing the risk of culture contamination, and no time delay is observed when acquiring the data, making them a great solution for real-time monitoring. Also, as non-invasive techniques, spectroscopic sensors do not interfere with the biological material, allowing to monitor *in vivo* cells and obtain information on their intracellular state, in addition to information on extracellular media (Marose *et al*., 1999; Ulber *et al*., 2003; Hantelmann *et al*., 2006; Glindkamp *et al*., 2009).

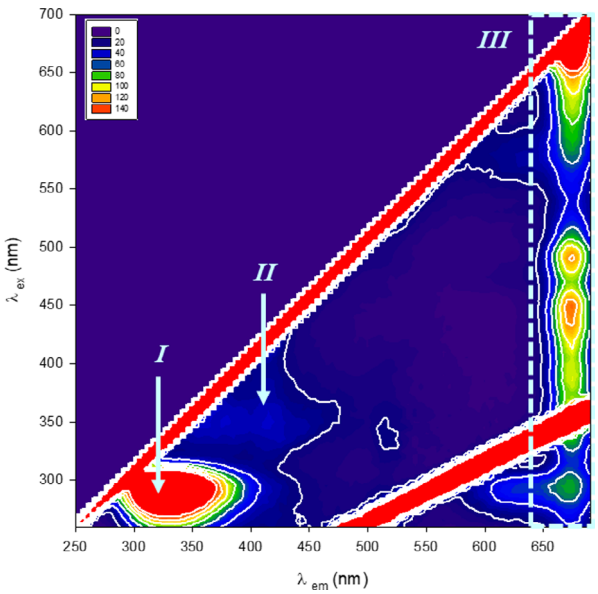

Fig. 1. Fluorescence spectra of Dunaliella salina microalgae. Emission wavelengths in the x-axis, excitation wavelengths in the y-axis, and fluorescence intensity in colour-grade scale. Three distinct fluorescence regions are identified: I – protein-like region (excitation 275 nm, emission 300–350 nm); II – humic compounds region (excitation 350 nm, emission 400 nm); and III – pigments band (emission higher than 650 nm).

The spectroscopic optical sensors can be divided into three categories based on the way they interact with the sample: (i) they can be used in combination with an indicator for specific molecules (usually fluorescent dyes) (Ulber *et al*., 2003); (ii) coupled to a biological receptor like a catalytic effect (enzyme-based) or comprise immune and gene sensors (antibodies) (Marose *et al*., 1999); (iii) or, in the most simple case, analyze the optical properties of the sample (Wolfbeis, 2005). This last approach is more adequate when aiming to monitor cell cultivation since no interaction with the culture is required, and several metabolites can be analyzed at the same time. Several spectroscopic methods, including infrared, Raman, and fluorescence spectroscopies, have been used for (bio)process monitoring, often in combination with optical fibres (Wolfbeis, 2005).

UV/Vis

Ultraviolet/visible (UV/Vis) has been, and still is today, widely used to monitor a wide number of parameters in various applications. UV/Vis is one of the most simple, universal, and inexpensive optical technique that allows high scanning speeds in the range from 200 to 780 nm (Ulber *et al*., 2003; Ryder, 2018; Singh *et al*., 2019). This technique has two major drawbacks: the lack of specificity and can only be used in liquid and homogenous samples (Ulber *et al*., 2003; Claßen *et al*., 2017). Although the presence of particles causes the light to scatter, this behaviour is frequently used as an advantage as optical density (OD), to quantify biomass concentration in turbid samples (Claßen *et al*., 2017). UV/Vis can be used to monitor large molecule groups such as proteins, as total protein concentration due to the typical absorption

between 260–280 nm. UV/Vis can also be used to monitor dissolved organic carbon (DOC) and different minerals in natural organic matter to assess water quality, and in wide applications in food industry, from roasting coffee to gin storage (Belay *et al.*, 2008; de Carvalho Polari Souto *et al.*, 2015; Govindaraj *et al.*, 2020; Xie *et al.*, 2021; Zhu *et al.*, 2021).

Infrared spectroscopy

Infrared (IR) spectroscopy provides information about the structural composition of the molecule (position, shape, and size) through the detection of the biological bonds C–H, N–H, O–H, and S–H. IR has been used in bioprocess monitoring for the measurement of several metabolites, such as ethanol, glucose, and fructose (Glindkamp *et al.*, 2009). IR has also been used for lipid research for the oil and fat industry, for example, to determine trans-isomers in fats (Laurens and Wolfrum, 2011). Because most bioprocesses occur in aqueous medium, and water is known to have a high IR absorbance for wavelengths higher than 2500 nm, IR spectroscopy can only be used with a short optical path length or in the range of near-to short-wave IR range (NIR), from 780 to 2500 nm (Glindkamp *et al.*, 2009).

The use of Fourier transform IR (FTIR) opened the possibility to use IR spectroscopy in more dilute products, increasing the range of opportunities in the food industry, such as dairy, and in dilute cellular cultivations, such as microalgae and cyanobacteria (Laurens and Wolfrum, 2011).

Near-infrared (NIR) has been developed as a rapid inexpensive method to monitor the chemical composition of food and feed. Like in FT-IR, the NIR absorption spectra are a result of the overtones and vibration combinations of the sample's chemical structure. However, NIR bands are broader and less defined and can be weak, similar, and/or overlap (Laurens and Wolfrum, 2011). Each wavelength can have contributions of several molecules present in the sample, and each molecule itself can absorb more than one wavelength. Therefore, it is necessary to apply advanced data analysis to obtain the information embedded in the spectra. Nevertheless, the restrictions of this technique also bring advantages. The low absorption coefficients enable a higher penetration depth, allowing the use of this technique in solids or turbid liquids, such as culture broths (Marose *et al.*, 1999; Ulber *et al.*, 2003; Glindkamp *et al.*, 2009; Henriques *et al.*, 2010).

An NIR spectrometer coupled with an optical probe was used to monitor cell density *via* light absorption (turbidity) or scattering in the visible and/or NIR range (Marose *et al.*, 1999). However, some of the bottlenecks need improvement: the inability to distinguish viable from non-viable cells, the narrow cell concentration range where it can be applied, and the sensitivity to different cell morphologies. Some products were monitored in fermentations, such as ethanol, glucose, glycerol, ammonia, or lactic acid, some in-/online, and some offline (Marose *et al.*, 1999; Claßen *et al.*, 2017).

NIR spectroscopy is currently used by several industries to check authenticity, for example in chicken meat (Parastar *et al.*, 2020) or to provide a biochemical fingerprint, as in tobacco, fishmeal, hazelnuts, and other dried fruits (Pannico *et al.*, 2015; Muresan *et al.*, 2016).

Raman spectroscopy

Unlike IR spectroscopy that is being used in industrial processes for several years, Raman spectroscopy is still in the stage of academic research (Claßen *et al.*, 2017; Schulz, 2018). Raman spectroscopy is based on the phenomena of shifted wavelength scattering of molecules excited with monochromatic light due to inelastic collisions of photons with the molecule (Ulber *et al.*, 2003). These collisions are dependent of the molecular composition of the sample under analysis. This technology does not require clear samples and enables the use of immersion probes, increasing the spectrum of usage in biotechnology industries (Claßen *et al.*, 2017; Ryder, 2018). Furthermore, the development of Fourier-transform allowed to considerably reduce acquisition times and photodecomposition. Nevertheless, the intensity of the signal acquired is very weak and difficult to separate from the scattering, and it cannot be used with strongly fluorescent samples (Ulber *et al.*, 2003; Singh *et al.*, 2019).

Nowadays, Raman spectroscopy is being researched to replace the standard procedures, such as GC or HPLC, for quality control and adulteration detection of several food products, or even pesticides (Shaw *et al.*, 1999; Schulz, 2018). Several authors described the use of Raman spectroscopy, together with advanced data analysis, to monitor several parameters within microbial cultivations, such as biomass concentration, substrate consumption (glucose, glycerol, ammonia), and product formation (penicillin, glutamine) (Marose *et al.*, 1999; Ulber *et al.*, 2001; Claßen *et al.*, 2017).

In-depth look into fluorescence spectroscopy

Principle

The phenomenon of light emission (luminescence) can be divided into two types, phosphorescence and fluorescence. The difference between them is the time in which the absorbed light is emitted. Fluorescence occurs when an excited singlet state returns to its ground state by the rapid emission of a photon

(Lakowicz, 2006; Ranzan *et al.*, 2012). For phosphorescence, the absorbed light can be stored and emitted between a few seconds or hours.

The first fluorescence sensors developed enabled only one wavelength of excitation and emission, meaning that only one fluorophore could be measured, restricting the use of this technology in complex processes. Later on, the development of multiwavelength fluorescence sensors made it possible to detect simultaneously several fluorophores in the same measurement, boosting the use of fluorescence spectroscopy as a scanning technique (Marose *et al.*, 1998; Ulber *et al.*, 2003; Glindkamp *et al.*, 2009; Ranzan *et al.*, 2012). The measurement of several emission wavelengths over a range of excitation wavelengths creates a two-dimensional excitation–emission matrices (EEMs), that can be plotted in three-dimensional graphs through the intensity recorded for each excitation–emission pair. (Tartakovsky *et al.*, 1996; Marose *et al.*, 1999; Lakowicz, 2006; Sádecká and Tóthová, 2007; Lenhardt *et al.*, 2015). Figure 1 shows the typical fluorescence spectra measured at-line during membrane harvesting of *Dunaliella salina* microalgae (at a cell concentration of 1.8×10^6 cells mL^{-1}), where the *x*-axis indicates the emission wavelength, *y*-axis indicates the excitation wavelength, and the intensity is represented by a color gradient defined in the legend.

Fluorescence spectroscopy is a noninvasive technique, with highly sensitive detection and specificity, able to detect instantaneously several fluorophores. These fluorophores can be divided into extrinsic and intrinsic ones. Extrinsic fluorophores are usually added to a sample that does not have fluorescence itself, such as fluorescein and rhodamine. Intrinsic fluorophores occur spontaneously in nature, such as NAD(P)H, chlorophyll, amino acids, cofactors, and vitamins (Tartakovsky *et al.*, 1996; Marose *et al.*, 1999; Podrazký *et al.*, 2003; Hantelmann *et al.*, 2006; Lakowicz, 2006; Ranzan *et al.*, 2012; Sá *et al.*, 2019, 2020; Gao *et al.*, 2021).

Fluorescence spectroscopy is sensitive to metabolites, intracellular and extracellular, and to medium composition. In biological systems, where the medium is characterized for having a rich composition, the interaction fluorophore-medium is rather complex. Parameters such as the polarity of the medium, fluorophore's structure, and the interaction between fluorophores and medium molecules can cause a shift in the spectra. Quenching is the phenomenon of masking the fluorescence intensity signal of natural fluorophores. Quenching can be collisional or static if the decrease in the fluorescence is achieved by contact with another fluorophore or when forming a non-fluorescent complex, respectively (Ulber *et al.*, 2003; Lakowicz, 2006). For this reason, the use of fluorescence to indirectly infer non-fluorescent compounds was reported since their presence can affect the fluorescence captured, and therefore, create a fingerprint in the fluorescence spectra (Ulber *et al.*, 2003; Sá *et al.*, 2020a, 2020b). A different phenomenon is the inner-filter effect, also mentioned as self-absorption, where the fluorescence light is absorbed by the fluorophore itself (Larsson *et al.*, 2007). Also, very dilute solutions will have a higher scatter interference in the EEMs because of the interaction between water and fluorophore molecules. Two types of scatter can be noticed: 1st and 2nd order Rayleigh, when the emission equals the excitation or two times the excitation, respectively, and Raman, with a shift to longer wavelengths called red shift (Zepp *et al.*, 2004; Bahram *et al.*, 2006).

Biochemical applications

The urge for an in-/online technology not only in the bioprocess industry but also in environmental and pharmaceutical applications accelerated the research and development of a system that could be coupled to a bioreactor, *via* optical fibre, and that could give a real-time view of the process.

Several research groups have studied the use of fluorescence spectroscopy as an online monitoring tool for fermentations. Most of the reported cases investigated the fluorescence of the reduced form of NAD(P)H, in which fingerprint is detected at an excitation wavelength of 340 nm and emission wavelength of 460 nm. NADH is a highly fluorescent molecule, and it is known that the fluorescence intensity signal has a good correlation with the biomass concentration and its metabolic state (Tartakovsky *et al.*, 1996; Marose *et al.*, 1999; Schügerl, 2001; Pons *et al.*, 2004). For that reason, fluorescence spectroscopy has been used to track physiological changes, such as the transition between bacterial aerobic and anerobic metabolisms (Glindkamp *et al.*, 2009), and the change between oxidative and oxidoreductive metabolism in yeasts (Hantelmann *et al.*, 2006). Fluorescence spectroscopy was also used to monitor biomass and substrate or product concentrations in *E. coli* and *S. cerevisiae* fermentations (Marose *et al.*, 1999; Podrazký *et al.*, 2003).

The flexibility of fluorescence spectroscopy is well-reported for the monitoring of a number of analytes, *via* direct or indirect correlations, such as proteins, vitamins, co-enzymes, glucose, ethanol, ATP, pyruvate, nitrate, or succinate (Shaw *et al.*, 1999; Ulber *et al.*, 2003; Hantelmann *et al.*, 2006; Glindkamp *et al.*, 2009; Ranzan *et al.*, 2012). DELTA Light & Optics (Lyngby, Denmark) developed the BioView™ and used to predict several cultivation parameters, such as enzyme activity, product formation, and substrate consumption, with the final goal of defining an optimal harvest time (Marose *et al.*, 1998; Pons *et al.*, 2004).

Fluorescence spectroscopy has also been studied as a monitoring tool in several fields of the food industry, such as the industrial downstream processing of sugar beet molasses (Ulber *et al.*, 2003), the characterization and classification of honey (Lenhardt *et al.*, 2015), or the detection of orange juice frauds (Ammari *et al.*, 2015). Other applications under study include *in situ* characterization of polycyclic aromatic hydrocarbons (PAHs) (Grundl *et al.*, 2003) or even as a sensor to detect illegal drugs (cocaine and marijuana) in street samples (Babicheno *et al.*, 2004), which shows the versatility of this technique. In the pharmaceutical field, fluorescence spectroscopy was studied as a monitoring tool for the physiological state of mammalian cell cultures, a platform to produce antibodies, blood, and growth factors or cytokines (Teixeira *et al.*, 2009). Also, several studies reported the potential of using this technique in wastewater treatment plants (Wolf *et al.*, 2001, 2005; Hambly *et al.*, 2010; Galinha *et al.*, 2012; Louvet *et al.*, 2013; Carstea *et al.*, 2016). Ranzan *et al.* stated that the application of this technology as a monitoring tool in biological systems proved to improve the ecological and economic management of the overall process under study (Ranzan *et al.*, 2012). More applications can be found in the review of Pons *et al.* (2004).

Pulse Amplitude Modulated (PAM) fluorometry has been used in the scientific fields of plants and microalgae to determine photosynthetic activity and detect physiological stress (White *et al.*, 2011; Zhao *et al.*, 2017). Through PAM measurements, it is possible to determine parameters that tightly correlate with the functional state of photosystems I and II, such as non-photochemical quenching (NPQ), maximum quantum efficiency (F_v/F_m), or light saturation (E_k). Some works reported the correlation between PAM measurements and stress indicators, such as lipid accumulation (White *et al.*, 2011; Zhao *et al.*, 2017). More about PAM fluorometry can be found elsewhere.

Although a major advancement was observed in the quality of the optical sensors used in spectroscopy methodologies, these sensors are still rarely used in the biotechnology industry, and most of the devices developed have been only used for research purposes. The main difficulties described for the restricted application of this technology to date are mostly due to interferences that influence the quality of the fluorescence spectra, such as turbidity, gas bubbles, and fouling (Tartakovsky *et al.*, 1996; Marose *et al.*, 1999; Ulber *et al.*, 2003).

Chemometrics: a brief description of the mathematics needed for spectra interpretation

A full fluorescence spectrum contains not only complex in-depth information about the natural fluorophores present in the sample but also the interferences between them and the environmental medium (Marose *et al.*, 1999; Schügerl, 2001). Therefore, chemometric methods are used to deconvolute the data within fluorescence matrices and to establish the relationship between them and the concentration of substrates and products to be monitored (Teixeira *et al.*, 2009; Henriques *et al.*, 2010; Galinha *et al.*, 2012, 2013).

The first records of chemometric methods were reported by Mandel in 1949, but only 25 years later, the name "chemometrics" was invented (Mandel, 1949). According to the definition of the Chemometrics society, chemometrics is "the chemical discipline that uses mathematical and statistical methods to design or select optimal procedures and experiments and to provide maximum chemical information by analyzing chemical data".

Multivariate analysis, such as principal component analysis (PCA), is often used to extract meaningful information from the spectra, resulting in the reduction of the size of the data set. For a n number of initial variables (X_1, X_2, …, X_n), n linear combinations are obtained, the so-called principal components (PCs), characterized for being uncorrelated and ordered according to the variance explained (the first PC explains the higher part of the variance, and smaller parts of variance are explained with subsequent components) (Sádecká and Tóthová, 2007; Leardi, 2008). The PCA defines the initial data set (the matrix X) as $\mathbf{X} = \mathbf{T}.\mathbf{PT} + \mathbf{E}$, where **T** is the score matrix (represents the objects in the new orthogonal space), **P** is the loading matrix (represents the relationships between the PCs and the original variables), and **E** is the error (residuals) matrix (contains the difference between the observed values and the ones modelled by the PCA).

Most of the chemometric applications in biochemistry are prediction models since the use of spectral measurements to replace time-consuming chemical and biological lab analysis is very appealing. In other words, chemometric models are often used to find correlations between a bio-/chemical variable (called dependent variable, y) and the data acquired from instruments such as a spectrofluorometer (independent variable, x) (Brereton *et al.*, 2018). This relationship established between the dependent variable, and a set of independent variables will be used as a linear polynomial prediction model, defined as $y = b_0 + b_1x_1 + b_2x_2 + ... + b_kx_k + f$, where b_0 is an offset, b_k ($k = 1, \ldots, K$) are regression coefficients, and f is the residual. To calculate b, the vector of the regression coefficients, several methods can be used: (i) multiple linear regression (MLR), that require uncorrelated spectral variables (x-variables), which can be hard to find in fluorescence spectra where collinearity is common; (ii) principal component regression (PCR), a MLR

analysis applied to PCA scores, where PCs that explain high variability in the spectra are correlated with the target properties (*y*); (iii) projection to latent structures (PLS), that find the correlations that best describe the highest covariance between spectral (*x*-variables) and target properties (y), expressed in a new basis (latent variables) (Tartakovsky *et al*., 1996; Marose *et al*., 1999; Ulber *et al*., 2001; Sádecká and Tóthová, 2007; Leardi, 2008; Glindkamp *et al*., 2009; Ranzan *et al*., 2012).

When the regression methods fail to develop a linear relation between inputs and outputs, the use of nonlinear methodologies is helpful in modeling more complex data, for example, multivariate additive PLS splines (MAPLASS) or artificial neural networks (ANN) (Yu *et al*., 2018). Briefly, ANN embodies a "machine-learning" algorithm that attempts to mimic the information processing found in the human brain, using artificial "learning-from-experience". Detailed information about ANN can be found elsewhere (Wolf *et al*., 2001; Lin *et al*., 2012; Oliveira *et al*., 2017; Yu *et al*., 2018).

The possibility of having a clear insight of the biochemical processes and reactions is a powerful tool for numerous industries. Workman (2002) presented several advantages of using chemometrics, among them: (i) allows the possibility of providing real-time and high-quality information from fewer data; (ii) improves the existing knowledge of the processes under study and enables the improvement of measurements; and (iii) requires low capital investment.

The combination of a high-sensitivity and resolution spectrophotometry, such as fluorescence spectroscopy, with chemometrics analysis for data pre-treatment and exploration, enriches the knowledge and control of the processes. In line with that, Process Analytical Technology (PAT) has been presented as a way to minimize variability in manufacturing processes, enabling their control. PAT is defined as a system to design, analyze, and control processes through timely measurements of key parameters (Simon *et al*., 2015). These parameters can be process parameters (as pH or temperature) and performance attributes, which in the case of biotechnology processes can be biomass concentration and product formation from chemical and/or microbiological methods and spectroscopic analyses.

Case-study: microalgae production

Microalgae are well-known photosynthetic microorganisms with the ability to produce a wide variety of metabolites with the use of sunlight and CO_2 fixation from the atmosphere or fume gas. When comparing microalgae to plant crops, they present several advantages: they have higher areal productivity and higher biomass production rates, they can grow in diverse and inhospitable environments, they do not compete for land, and some species can grow in seawater (Zeng *et al*., 2011; de Vree *et al*., 2016).

However, nowadays, microalgae production is far from terrestrial crops since the production capacity of microalgae is still performed in niche markets for high-value products. Their industrial production costs need to be reduced, and the scale needs to increase to make this industry more competitive (Posten, 2009; Wijffels *et al*., 2010). In Europe, most of the industrial cultivation facilities are based in closed photobioreactors (71%), against open ponds (19%), and fermenters (heterotrophic biomass production) (10%) (Fig. 2) (Araújo *et al*., 2021).

Since the growth and productivity of microalgae cultivation is so tightly correlated with light conditions, better engineering solutions must be developed regarding light distribution, mass transfer, and hydrodynamics (Posten, 2009; de Vree *et al*., 2016; Esposito *et al*., 2017; Araújo *et al*., 2021). Operational costs are still too high and dependent on the local conditions and the metabolites produced for commercialization (Posten, 2009; Araújo *et al*., 2021).

By minimizing the production and processing costs of microalgae biomass, the cost of microalgae products will

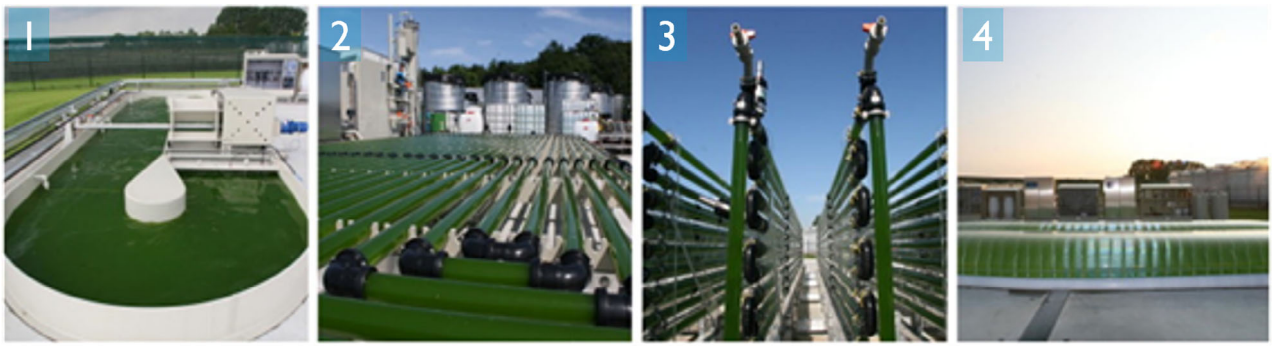

Fig. 2. Photobioreactor's configurations, available at AlgaePARC, at Wageningen University and Research (from right to left): (1) raceway pond, (2) horizontal tubular reactor, (3) vertical stacked tubular reactor, and (4) flat panels.

decrease. The new technologies developed must be easily scalable and competitive in operational and investment costs. To increase the economical yield of this industry, a biorefinery approach has been proposed where, instead of focusing on a specific product, a portfolio of products needs to be extracted, separated, and purified according to its end market (Wijffels *et al.*, 2010).

In a microalgae biorefinery production, the entire value chain is connected. Higher product yields need high biomass productivity, high biomass harvesting, and processing efficiency. Microalgae cultivation is mostly done in very diluted biomass concentrations, from 0.2 to 2 g l^{-1}, to avoid large dark zones in the cultivation systems, which leads to low productivities (Bilad *et al.*, 2014; de Vree *et al.*, 2016). This low biomass concentration with consequent large cultivation volumes leads to high dewatering costs. The most common technology used for dewatering microalgae biomass is centrifugation, which is a very energy-demanding technology (Bhave *et al.*, 2012). To reduce harvesting costs, two-step harvesting processes are being studied, for example, using membrane filtration as a primary dewatering step before centrifugation (Bhave *et al.*, 2012; Monte *et al.*, 2018).

Microalgae cultivation can be a complex system with several biological, fluid dynamics, environmental, and nutritional conditions playing a crucial role in the productivity and viability of the industrial process. Therefore, the development of prediction models that allow in-/online monitoring of substrates and products is fundamental for process control, enabling the possibility to take decisions and actions in real-time (Esposito *et al.*, 2017).

Cell concentration

In the current industrial scenario, most microalgae products are sold as whole biomass powder, making total biomass a key parameter to control process efficiency. During cultivation at industrial scale, too high or too low biomass concentration can influence several biological parameters. Low concentrations can result in inefficient light absorption or photoinhibition, while high concentrations result in dark regions in the bioreactor that provokes endogenous respiration (de Vree *et al.*, 2016).

Biomass concentration can be monitored using different parameters, such as optical density (OD), dry weight (g l^{-1}), and cell concentration (cells l^{-1}), most of them measured offline. Measuring cell concentration is the most accurate method to evaluate microalgae growth since some cultivation conditions also induce several biological changes, such as coloration or accumulation of fatty acids. For example, as mentioned by Janssen *et al.* (2018), accumulation of lipid bodies due to nitrogen depletion leads to an increase of the dry weight while the cell concentration reaches a plateau. Experiments using a day/night cycle lead the biomass to follow circadian rhythms, which means that cell size increases during the day while the cell concentration increases during the night (due to cell division), leading to misleading OD measurements (Fabregas *et al.*, 2002; de Winter *et al.*, 2013).

Monitoring cell concentration using fluorescence spectroscopy was reported in several studies in different microalgae and for different production processes, for example, cultivation and harvesting (Sá *et al.*, 2017, 2019, 2020; Monte *et al.*, 2018; Gao *et al.*, 2021). In all these studies, fluorescence spectroscopy, coupled with different modeling techniques, proved to be a robust tool in a wide range of biomass concentrations disregarding the microalgae size or biological state (Sá *et al.*, 2019). The most promising result was reported by Gao *et al.* (2021). For the first time, using only fluorescence spectroscopy EEMs, the authors develop one single prediction model able to be used for cell concentration prediction of different microalgae species (tested with *Tisochrysis lutea* and *Phaeodactylum tricornutum)*, revealing the high potential of this technique to be used in microalgae production (Gao *et al.*, 2021). This is advantageous from the typical OD measurement that provides different results for different cell morphologies.

Other spectroscopies have also been used to monitor cell concentration during microalgae cultivation. For example, direct coupling of an optical probe and Raman spectroscopy were reported to successfully monitor cell concentration of heterotrophic cultivation of *Auxenochlorella protothecoides* (Nadadoor *et al.*, 2012).

Pigments

While cell concentration can be a rather simple measurement, assessing metabolite content is a much laborious, expensive, and time-consuming work, involving extraction steps and chromatographic techniques. Therefore, the development of prediction models for pigments, able to be used online, is of extreme importance for microalgae industrialization.

Chlorophyll is the most abundant light-harvesting pigment in nature and is a molecule well-studied for its potential use in several fields. In the feed and food supplement industry, chlorophyll is relevant due to its antioxidant properties. Because of its bright green color, it is also an appealing dye for the food and paint industries (Rodrigues *et al.*, 2015; Chew *et al.*, 2017). Chlorophyll content in the microalgae is tightly correlated with light intensity and circadian rhythms. It was reported that chlorophyll content increases during the light period and starts to decrease with the beginning of a dark period

(Fabregas *et al*., 2002; de Winter *et al*., 2013; Braun *et al*., 2014). This phenomenon is explained by the fact that the cell division mechanism is favorable in the dark period, where the chlorophyll content of the "adult" cell is divided between the new "daughter" cells (Fabregas *et al*., 2002). Moreover, microalgae are known for their ability to adapt their photosynthetic apparatus to different light conditions, a process called photo acclimation. High light intensities reduce chlorophyll content to protect the cell; while low intensities induce the photosynthetic apparatus to synthesize chlorophyll and provide the cell with higher light-harvesting capacity (Dubinsky and Stambler, 2009; Janssen *et al*., 2018). Fluorescence spectroscopy was already used to monitor and model the content of chlorophylls in *Dunaliella salina* and in *Nannochloropsis oceanica*, separately (Sá *et al*., 2019, 2020). Other reports show the potential of this technique to distinguish several chlorophylls (*a*, *b*, and *c*) and their degradation products (pheophytin *a* and *b*, and pheoporphyrin *c* in acetone: water model-solutions (Moberg *et al*., 2001) and the possibility to estimate algal blooms (Gregor and Maršálek, 2005; Ziegmann *et al*., 2010). Shin *et al*. (2015) described the development of a portable and low-cost fluorescent sensing by coupling a microfluidic chip with multiple light-emitting diodes for excitation and a photodetector to measure the fluorescence signal from a microalgal sample. The development of this technology is of great importance for the microalgae industry, even if some improvements and adjustments are needed to make it more accurate and for multi-detectable purposes since it can be easily incorporated in industrial microalgae production systems.

Some microalgae are well-known for their ability to accumulate carotenoids, a family of light-harvesting pigments and reactive oxygen species (ROS) scavengers, that act as non-photochemical quenching, which means they can absorb the excess light preventing damage to the photosynthetic apparatus. The global market for carotenoids was nearly $1.5 billion in 2017 and should reach $2.0 billion by 2022 (McWilliams, 2018).

For example, the industrial production of *D. salina* is one successful case of microalgae production. This halotolerant microalga can produce high contents of carotenoids, approximately 10% of its total dry weight, under stress conditions such as high salinity, high light intensity, nutrient depletion, or extreme temperatures (Fig. 3) (Ben-Amotz, 1983; Borowitzka *et al*., 1990; Lamers *et al*., 2012).

The cultivation systems more often used for *D. salina* cultivation are large unstirred open ponds or paddlewheel stirred raceways. A two-step cultivation is commonly applied: the first stage, a "green" phase, where growth is done under optimal conditions; a second stage, the "orange" phase, where the culture is submitted to stress factors and the carotenoid production is enhanced (Wichuk *et al*., 2014).

In a previous study, fluorescence spectroscopy was also used to monitor simultaneously different carotenoids, such as zeaxanthin, α-carotene, all-trans-β-carotene, and 9-cis-β-carotene in *D. salina* in different biological states, from non-stressed ("green") to stressed ("orange"), induced by nitrogen depletion or increased salinity (Sá *et al*., 2019). Although the analytical methodologies commonly used for pigment analysis (such as HPLC) are accurate and able to assess different compounds simultaneously, they are expensive, time-consuming, and require a laborious extraction step. The use of fluorescence spectroscopy, coupled with appropriate mathematical tools, enables the possibility of having several and repeated measurements at real-time, a motivating advantage for industrial production of these high value and in high demand compounds.

Other microalgal pigments were reported in the literature as an alternative source for the current production. For example, fucoxanthin has gained high attention due to its antioxidant, anti-obesity, and antidiabetic

Fig. 3. Dunaliella salina: "green" cells, rich in chlorophyll (left), and "orange" cells, rich in carotenoids (right).

Fig. 4. Tisochrysis lutea: different biomass concentrations, from more diluted (left) to more concentrated (right), in pilot-scale outdoor production at AlgaePARC (Green Wall Panel® III, F&M Fotosintetica & Microbiologica S.r.l., Italy).

properties, possible to be used in cosmetics, pharmaceuticals, and nutraceuticals (Guedes *et al.*, 2011; Fung *et al.*, 2013; Maeda *et al.*, 2018). At the moment, fucoxanthin is mainly extracted from seaweed, but several microalgae can accumulate higher concentrations, such as the marine brown species *T. lutea* and *P. tricornutum* (Gao *et al.*, 2020). Microalgal fucoxanthin can be stimulated by the self-shading effect of cells or low light intensity as a strategy to absorb sufficient light for photosynthesis (Faraloni and Torzillo, 2017). It is reported that *T. lutea* can accumulate up to 1.82% of its dry weight (DW) (Kim *et al.*, 2012; Mohamadnia *et al.*, 2020), while *P. tricornutum* can accumulate as much as 5.92% DW (McClure *et al.*, 2018). Therefore, both microalgae are promising candidates for fucoxanthin industrial production. Recently, Gao *et al.* (2021) reported the possibility of monitoring fucoxanthin content in these two different microalgae, in a pilot-scale production (Fig. 4), using only one prediction model, leveraging the use of fluorescence spectroscopy as a monitoring tool to a non–species-dependent technology.

Raman spectroscopy was used to determine the carotenoid profile in snow algae and cyanobacteria (Osterrothová *et al.*, 2019; Nekvapil *et al.*, 2021). Both works show promising results to quantify carotenoids, although the method was applied for single-cell analyses and not as a cultivation sensor. Also, the exact identification of the carotenoids still required calibration with an offline method. This technique was also used to monitor *Phaffia rhodozya*, a carotenoid-producing fermentative yeast, by direct coupling of an optical probe into the fermentation vessel. The authors took advantage of the high fluorescent signal of the carotenoids in the Raman spectra and did not use chemometric models (Cannizzaro *et al.*, 2003).

Lipids

Several microalgae are reported for their potential to accumulate high content and/or high-quality lipids. When cultivated under optimal growing conditions, most of the lipids in microalgae are present in the cellular and plastid membranes (Li-Beisson *et al.*, 2019). However, under stress growing conditions, some microalgae, like *Nannochloropsis* species, can accumulate up to 45% of their dry weight in triacylglycerol (TAG) (Fig. 5) (Ma *et al.*, 2016; Janssen *et al.*, 2019).

According to the cell location where the lipid is accumulated and its profile, the final destination of the lipid-enriched biomass can vary from feed or food supplements to biodiesel production (Caballero *et al.*, 2003; Draaisma *et al.*, 2013; Li-Beisson *et al.*, 2019). Fatty acids can be classified into saturated and unsaturated, according to the absence or presence of chemical double bonds. Biomass produced for the feed or food supplement industries is desired to be rich in unsaturated fatty acids, such as omega-3 fatty acids such as EPA (eicosapentaenoic acid) or DHA (docosahexaenoic acid) (Janssen *et al.*, 2019; Li-Beisson *et al.*, 2019). For biofuel applications though, the biomass has to fulfil ignition and combustion performance quality parameters, directly correlated with saturated and unsaturated content (Nascimento *et al.*, 2014; Ma *et al.*, 2016).

Due to the potential of fluorescence spectroscopy to identify natural fluorophores (intra or extracellular) and the relations between these and non-fluorophores, such as lipid molecules (Sá *et al.*, 2020a, 2020b) reported the use of this technology to monitor the lipid content in *N. Oceanica* not only lipid classes (saturated and unsaturated) but also specific fatty acids such as EPA.

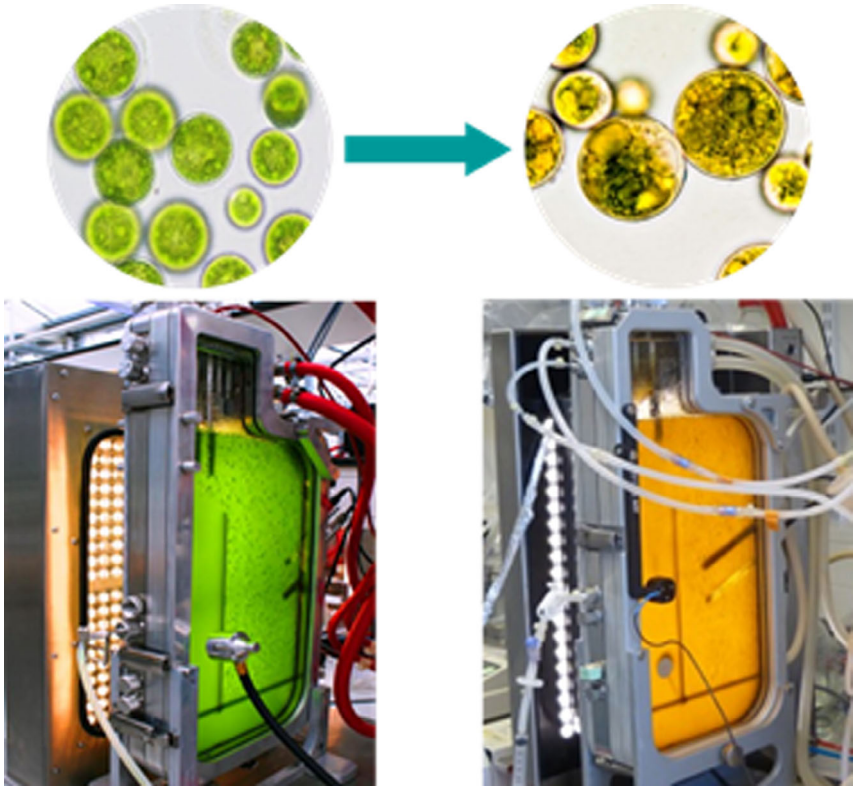

Fig. 5. Nannochloropsis oceanica: biomass with low lipid content (left) vs. high lipid content (right) (Labfors 5 Lux, Infors HT, Switzerland).

Additionally, it was shown that fluorescence-based models could also be relevant to EPA location between the neutral and polar fractions (Sá *et al*., 2020), indicating once more the potential to assess lipid quality in microalgae.

The degree of lipid unsaturation and TAG accumulation was also studied using other spectroscopic methods. For example, oil content in *A. protothecoides* was monitored online by Raman spectroscopy, directly in the fermentation vessel (Nadadoor *et al*., 2012). Detailed information about the use of Raman, FTIR, and NIR spectroscopy can be found somewhere else (Han *et al*., 2011; Laurens and Wolfrum, 2011; Wu *et al*., 2011; Nadadoor *et al*., 2012; Wang *et al*., 2014; Jaeger *et al*., 2016; Esther Elizabeth Grace *et al*., 2020). However, most of these studies were performed at single-cell level.

Future perspectives

Fluorescence spectroscopy, unlike other spectroscopy technologies, is still mainly confined to the lab environment. Its direct use outside academia is only a reality for chlorophyll content measurement through a narrow pair of excitation–emission wavelengths. And, as is shown in this review, fluorescence spectroscopy has a much higher potential than just using a few excitation–emission wavelength pairs. The possibility of obtaining an excitation–emission matrix increases the amount of information acquired, working as a fingerprint technique for the presence of natural fluorophores abundant in microalgae. Also, with the ability to take advantage of interferences, it is possible to infer non-fluorescent compounds, increasing the range of applications of fluorescence spectroscopy as an online monitoring tool. Since each measurement can take up to 5 min, it is adequate for real-time process control. This technique is a promising solution for monitoring up and downstream processes, different biological parameters simultaneously, and can be used in the production of specific added-value compounds from different microalgae species. Compared with other spectroscopic methods, fluorescence spectroscopy can detect milli- to-micromolar concentrations of some analytes in liquid samples without the need for sample pre-treatment.

To impulse the transfer to an industrial scenario, several suggestions are here proposed:

1. Calibrate the monitoring tool focusing on the product of interest instead of the microalgae of origin: although some initial work is already reported in the literature, the most common approach is still species-

centered. The development of models able to predict the same product or physiological parameter in different microalgae would be of great interest to the industry. It is common practice in several microalgae-producing companies to cultivate different species according to the season of the year, known as "winter" and "summer" species. Nevertheless, the target compounds of the microalgae biorefinery do not always change. Therefore, the prediction tool could be developed having in consideration the final product, the value-added metabolite of interest, or a specific physiological parameter, such as cell concentration.

2. Simplification of the spectrofluorometer and adaptation to industrial scale production: Fluorescence spectroscopy could be applied today in microalgae production if some improvements are made. After identifying the regions of the spectra that are relevant to monitor the target parameters, it would be a great advantage to simplify the acquisition equipment by selecting and tuning only the wavelengths needed. Not only the time per analysis would decrease but also the cost of the equipment acquisition and maintenance.

For small-scale cultivation systems, coupling several optical fibers from different PBRs to the same spectrophotometer could be a solution. However, the optic fibers currently available have up to 2 m length, decreasing the flexibility of this solution.

The adaptation of fluorescence spectroscopy will depend on the main goal of the company. For example, if the goal is to monitor cell concentration and specific metabolites during one step of the process, only one optical fiber per cultivation system would be needed. For small-scale cultivation systems, coupling several optical fibers from different PBRs to the same spectrophotometer could be a solution. A switch-box needs to be developed and optimized to allow the acquisition of signals from different PBRs simultaneously, and the overall cost increases per price of each optical fiber. The price of the spectrofluorometers and the optical fiber probes is highly dependent on the technology used to generate the fluorescence signal and the sensitivity of the signal acquisition. If the equipment works with a wide range of wavelengths, the complexity of the equipment increases as well as the price. The same can be said for the optical fiber probes. A lab-bench size spectrofluorometer, equipped with a monochromator, the most complete and sensitive system, can cost between 15.000 € and 20.000 €. An optical fiber with 1.5 m can cost around 2.000 €, and the system to connect the fiber to the spectrofluorometer around 8.000 €. The price and sensitivity of an optical fiber are also dependent on its length.

A better approach, also more adequate for larger-scale production PBRs, would be to develop simpler individual spectrofluorometer per PBR. Developments on miniturizing spectroscopy have been accelarating, opening new opportunities for online monitoring. An example of that, is the development of smaller OD sensors described in Sandnes *et al*. (2006) (Sandnes *et al*., 2006). Nevertheless, it is important to consider that an adaptation of the spectrophotometer sensors to the PBRs still needs to be designed.

There are advantages and disadvantages for each solution, and each microalgae production facility should carefully consider them according to their monitoring needs.

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Conflict of interest

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

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