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Review

On-Line Monitoring of Biological Parameters in Microalgal Bioprocesses Using Optical Methods

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Abstract: Microalgae are promising sources of fuels and other chemicals. To operate microalgal cultivations efficiently, process control based on monitoring of process variables is needed. On-line sensing has important advantages over off-line and other analytical and sensing methods in minimizing the measurement delay. Consequently, on-line, in-situ sensors are preferred. In this respect, optical sensors occupy a central position since they are versatile and readily implemented in an on-line format. In biotechnological processes, measurements are performed in three phases (gaseous, liquid and solid (biomass)), and monitored process variables can be classified as physical, chemical and biological. On-line sensing technologies that rely on standard industrial sensors employed in chemical processes are already well-established for monitoring the physical and chemical environment of an algal cultivation. In contrast, on-line sensors for the process variables of the biological phase, whether biomass, intracellular or extracellular products, or the physiological state of living cells, are at an earlier developmental stage and are the focus of this review. On-line monitoring of biological process variables is much more difficult and sometimes impossible and must rely on indirect measurement and extensive data processing. In contrast to other recent reviews, this review concentrates on current methods and technologies for monitoring of biological parameters in microalgal cultivations that are suitable for the on-line and in-situ implementation. These parameters include cell concentration, chlorophyll content, irradiance, and lipid and pigment concentration and are measured using NMR, IR spectrophotometry, dielectric scattering, and multispectral methods. An important part of the review is the computer-aided monitoring of microalgal cultivations in the form of software sensors, the use of multi-parameter measurements in mathematical process models, fuzzy logic and artificial neural networks. In the future, software sensors will play an increasing role in the real-time estimation of biological variables because of their flexibility and extendibility.

Keywords: microalgal cultivations; on-line monitoring; optical sensors; biological variables; software sensors

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1. Introduction

Microalgal biomass contains significant amounts of valuable components including lipids, proteins, carbohydrates, pigments and vitamins that can be separated and upgraded to various products in the biofuel, food, fodder, cosmetic and pharmaceutical industries [1,2]. Long chain polyunsaturated fatty acids produced by microalgae play a significant role as health food supplements [3].

Efficient medium- and large-scale microalgal cultivations require on-line monitoring methods as the bases for process control, not only of standard process variables such as temperature and pH but of products in the biological phase as well. On-line sensing has important advantages over classical off-line analytical methods: no sampling and sample processing is necessary and measurement results can be transmitted to controllers in real

time or with only a slight delay. Minimizing the measurement delay is of high importance for process control, and thus, on-line and in-situ sensors are preferred that employ, in closed photobioreactor cultivations, non-invasive measurement principles to avoid contamination. Optical sensors thus occupy a central position as they can be constructed to be non-invasive.

The terms on-line, in-line, at-line, off-line and in situ define the manner of measurement and sensor placement in the process. Definitions of the terms on-line, in-line, at-line, off-line and in situ differ slightly between different industrial branches [4–7]. In an on-line measurement setup, either the sample is drawn from the process and not returned to the process stream [6] or the sensor is placed in a continuous bypass [7]; in an in-line setup the sample is analyzed within the continuous stream flow and not removed from it; in an at-line setup the sample is removed from the process, isolated from and analyzed in close spatial proximity to it; in an off-line setup measurements are carried out in a separate lab, utilizing a discrete sample; and in an in-situ setup the sensor is placed in the reactor vessel itself and is continuously in contact with the content [6]. Generally, on-line and in-line methods differ from the off-line and at-line methods in the time in which information about process or material properties is obtained compared with the time in which these properties change [5]. With on-line and in-line methods, the measurement process is faster than the change in the system properties, while with at-line and off-line methods, the measurement process can be slower than those changes. It is also easier to automate on- and in-line methods, so these analyses permit continuous process control. At-line and off-line analyses are characterized by manual or automated sampling followed by discontinuous sample processing, measurement and evaluation. In general, continuous process control with at-line or off-line sensing is not possible.

Process variables requiring monitoring in microalgal cultivations can be divided into three groups [8]:

- Physical: light energy supply, temperature, mixing intensity, light frequency within the culture;
- Chemical: pH, pO_2, pCO_2, N, P, other nutrients, extracellular products, chemical contaminants;
- Biological: biomass concentration and composition (presence of intracellular products, mostly lipids and pigments), presence of other biological species, physiological state, photosynthetic efficiency (PE) (from which biomass yield on light energy can be derived), cell morphology.

Certain monitoring requirements in microalgal cultivations are distinct from those in most biotechnological processes:

- Monitoring of light in phototrophic microalgal cultivations; and
- Biological variables in microalgal cultivations are almost always intracellular products (lipids, carbohydrates, proteins) that are produced mostly in the stationary phase of the cultivation. Furthermore, there is a need to monitor harmful or competing biological contaminants (algae, pathogens, herbivores) in outdoor cultivations in open photobioreactors.

On-line monitoring of physicochemical variables in microalgal cultivations (temperature, pH, pO_2, pCO_2, inorganic nutrients, light intensity) is already available using standard sensors employed in the chemical and bioprocess industry: thermoelements, electrodes (pH, dissolved O_2 and CO_2, inorganic nutrients), gas analyzers (gaseous O_2, CO_2) and, specifically for phototrophic microalgal cultivations, measurement of light intensity with quantum sensors or dosimeters [9,10]. What remains is the monitoring of biological parameters/the biological phase, which is much more difficult because almost all the desired products in microalgal cultivations are intracellular [11]. In contrast to sensors measuring physicochemical variables, on-line sensors measuring biological variables as concentrations of lipids, carbohydrates and proteins or physiological variables as photosynthetic efficiency are in an earlier developmental stage.

In the last decade, evolution in the implementation of existing and established methods (e.g., optical spectroscopy) to the monitoring of the biological phase in microalgal cultivations has occurred and some new approaches (implementation in microfluidic devices, laser reflectance, hyperspectral imaging) have been developed and examined. The support in signal processing using chemometric models and machine learning has also grown. In this review, these developments are summarized with focus on monitoring the biological phase (biomass concentration and composition, physiological state, morphology, biological contaminants) using non-destructive, real-time, on-line, or in-line methods that avoid contamination of the running cultivation in closed photobioreactor systems, minimize measurement delays and thus supply the information required for successful process control without physically affecting the cultivation. Optical methods, including microscopy, spectroscopy of absorption, reflectance and scattering, and multispectral/hyperspectral imaging form the core of available methods. Non-optical methods, including nuclear magnetic resonance spectroscopy (NMR) and measurement of capacitance, impedance, or dielectric effects, have been also used for on-line monitoring of biomass components. Physical sensors built on these physical principles supply measurement signals that are further processed by software sensors, i.e., chemometric methods, image processing, various mathematical models, and artificial neural networks, or a combination of those, to yield meaningful process values enabling to estimate concentrations of desired cultivation products. The massive low-cost computing power available today provides fast data acquisition even when the raw hardware sensor data must be extensively processed.

Reviews focusing specifically on possibilities of monitoring biological variables in microalgal cultivations are already numerous, but they primarily present off-line methods, sometimes including automated methods implemented at-line [12–14]. In contrast, this review focuses both on optical hardware sensors of biological variables that can be employed in the on-line or in-line mode and on software sensors using signals from hardware sensors of all suitable types. Most sensors of biological variables mentioned in the review are in the research and laboratory stage, while some have been used in a pilot-scale cultivation.

This review is divided into several sections. First, measurement methods used for on-line monitoring of biological variables are reviewed in Section 2, arranged according to the measured variable and the employed method: biomass concentration measured by optical density, fluorescence, color and reflectance; mixed culture discrimination, cell number concentration, cell morphology, culture health monitoring, microalgal species identification, photosynthetic efficiency and quantum yield, and finally, biomass composition in terms of lipid, carbohydrate, pigment and protein concentration. In Section 3, high-throughput methods are briefly mentioned because of the possibility of converting them into true on-line methods. Section 4 deals with computer-aided on-line monitoring in the form of software sensors, reviewing approaches based on observers, Kalman filters, machine learning, artificial neural networks and chemometric models. Perspectives of the on-line sensing in microalgal cultivations are discussed in Section 5 and conclusions are drawn in Section 6.

There are quite a few publications especially in the field of software sensors where several biological variables are estimated simultaneously from the same set of measurement data. Such publications are then cited several times in the respective section. The same principle applies to publications cited more than once, both in sections covering the measurement method and in sections covering the data processing approach.

2. Measurement Methods Used for On-Line Monitoring of Biological Variables

In this section, on-line measurement methods are listed, with several exceptions where an off-line measurement method is mentioned, mostly because of the possible future on-line implementation. Monitoring methods, the corresponding monitored variable(s), and the sensor type are summarized in Table 1. The reported accuracy of these methods, together with the biological variable measured, measurement method and measurement conditions and limitations, is shown and compared in Table 2. Biomass concentration and cell count

can be monitored using optical density, color analysis, fluorometry, spectral methods or permittivity with accuracy from about R^2 = 0.90 to R^2 = 0.99. Biomass components as lipids, pigments and proteins can be monitored using NMR, PAM (quantum yield), spectral methods, fluorometry and ISM with comparable accuracy with that of biomass and cell count but with more complex and more expensive instrumentation, especially lipids with NMR and PAM and fatty acids with ISM.

The most often encountered process variable estimated in microalgal cultivations is the biomass concentration, determined using several physical measurement signals: turbidity, absorbance (optical density) at a predefined wavelength or as a spectrum, reflectance, color analysis (red–green–blue; RGB), IR spectroscopy and fluorescence, sometimes combined with a software sensor (observer). Other estimated variables are the pigment and lipid content, and for their estimation, physical measurements of cell count, nitrate and glucose concentration are employed, complemented by process signals obtained by various methods as measurement of turbidity, IR spectrum and fluorescence, RGB imaging and transmission spectra, NMR spectroscopy, fluorescence, hyperspectral or RGB imaging, and dielectrophoresis.

Table 1. Monitoring method of biological variables in microalgal cultivations implemented on-line or with on-line potential. (PAM: pulse amplitude modulation, a fluorescence technique; ANN: artificial neural networks; ISM: In-Situ Microscopy).

Monitoring Method	Monitored Variable (Concentration)	On-Line/Off-Line	Sensor Type	Comment	References
OD, turbidity (single wavelength)	Biomass	On	Self-constructed 560 nm Amphenol TSD-10 730 nm Commercial 880 nm	Flow-through cell	(1) [15–18]
OD (multiple wavelength)	Biomass Growth phase Chlorophyll	On Off	(1) OD self-constructed LED 400, 750, 850 nm Laser 650, 685, 780 nm (2) 550, 665, 750 nm	(1) Flow-through cell	(1) [19,20] (2) [21]
Reflectance	Contamination Biomass Cell count	On Off	(1) Spectrometer (2) (a) Reflectance probe (2) (b) Spectroradiometer	Contamination on-line	(1) [22] (2) (a) [23,24] (2) (b) [25]
Color analysis (RGB)	Biomass	On Off	(1) Commercial color sensor (2) CCD camera, Webcam	(1) Flow-through cell	(1) [26] (2) [15,27–29]
Hyperspectral (Absorbance/transmittance spectrum)	Biomass Cell count Lipids Carotenoids	Off	(2) (a, c) Spectral camera (2) (b) Spectroradiometer (2) (d) Spectrometer		(2) (a) [30] (2) (b) [25] (2) (c) [31] (2) (d)[32]
ISM	Cell morphology Lipids	On Off	(1) In-Situ Microscope (2) In-Situ Microscope, holographic microscope		(1) [33] (2) [34]
Chlorophyll fluorometry	Protein Biomass Contamination	On Off	(1) LEDs/Photodiode (2) (a) Fluorometer (2) (b) 2D-Fluorometer	Single/multiple excitation ANN Chemometric model	(1) [35] (2) [36–38] (2) (a) [39] (2) (b) [40,41]
PAM fluorometry	Quantum yield (Photosynthetic efficiency) Contamination	On Off	PAM fluorometer	Stress detection (1) Light adapted except [42] (2) Dark adapted	(1) [42–46] (2) [47]

Table 1. *Cont.*

Monitoring Method	Monitored Variable (Concentration)	On-Line/Off-Line	Sensor Type	Comment	References
2D-fluorometry	Biomass Nitrate Cell count Cell viability Fatty acids Lipids Pigments	Off	2D fluorometer with a cuvette or with a fiber optics probe	Chemometric models	(2) [40,41,48–50]
NMR	Lipids	On	Benchtop NMR in a bypass	Expensive instruments	(1) [51–55]
Dielectric spectroscopy, dielectrophoresis, capacitance, impedance, permittivity	Viable cell concentration Lipids	On	(1) Commercial probe (2) Microfluidic device		(1) [15,56] (2) [57]
Microfluidic implementation	Lipids	On Off	(2) (a) PAM fluorometer (2) (b) Permittivity		(2) (a) [58] (2) (b) [57]
Mass spectrometry	Contamination	On	TOF mass spectrometer	Grazer detection Expensive instruments	(1) [59]

(1) On-line implementation. (2) On-line implementation possible.

Table 2. Accuracy of the reviewed methods where available in the original article. Included are only methods not using software sensors; these are shown in Section 4. (DWC: dry weight concentration (of biomass); OD: optical density; V_{out}: sensor output voltage; NTU: nephelometric turbidity unit; r, g, b: intensities of the red, green and blue components; T_{751}, T_{676}: transmittance at 751 and 676 nm; Chl a: chlorophyll a; CC: cell count; HR: hyperspectral reflectance; EC: extinction coefficient; OD_{560}: optical density at 560 nm; $\Delta\varepsilon$: change in permittivity; QY: quantum yield; ISM: In-Situ Microscope; DHA: docosahexaenoic acid; R^2: coefficient of determination; r: Pearson correlation coefficient).

Biological Variable	Measurement Method	Method Accuracy	Limitations/Conditions	Reference
Biomass	OD, turbidity	OD/DWC: R^2 = 0.81–0.96 [19] Vout/OD: R^2 = 0.95 [16] NTU/DWC: R^2 = 0.88–0.93 [18] OD/DWC: R^2 = 0.88–0.92 [18] OD/DWC: R^2 = 0.99 [20]	PBR bypass	[19] [16] [18] [20]
Biomass	Color analysis (RGB)	OD/DWC: R^2 = 0.998 [26] (r,g,b)/DWC: R^2 = 0.97–0.99 [28] (r,b)/DWC: R^2 = 0.90–0.96 [29]	PBR bypass [26] Open container/biofilm, suspension [28] Open container/suspension [29]	[26] [28] [29]
Biomass	Transmittance spectrum	DWC/T_{751}/T_{676}: r = 0.51–0.93 [30]	Microwells	[30]
Biomass	Chlorophyll fluorometry	DWC/Chl a fluorescence: r = 0.95 [35]	Fiber probe in PBR bypass	[35] [39]

Table 2. *Cont.*

Biological Variable	Measurement Method	Method Accuracy	Limitations/Conditions	Reference
Cell count	Transmittance spectrum	CC/T751/T676: r = 0.85–0.96 [30]	Microwells [30]	[30]
	Hyperspectral reflectance, EC	CC/HR,EC: R^2 = 0.99 [25]	Open container [25]	[25]
	Permittivity	CC/OD560: R^2 = 0.992–0.999 [56]	Flask bypass [56]	[56]
	Chlorophyll fluorometry	CC/Chl a fluorescence: r = 0.92 [35]	Fiber probe in PBR bypass [35]	[35]
Viable cell count	Permittivity (ε)	OD560/Δε: R^2 = 0.99 (calibration) OD560/Δε: R^2 = 0.77 (cultivation)	Commercial probe	[56]
Lipids	NMR	Algal lipids/NMR signal: R2 > 0.99 [55]	PBR bypass [55]	[55]
	Quantum yield ($\Delta F'/F_m'$)	Algal lipids/NMR signal: R^2 = 0.99 [52]	Bleed [52]	[52]
	NIR spectrum	Lipids as %DW/QY($\Delta F'/Fm'$): r = −0.96 [46]	In-situ fiber [46]	[46]
		Lipids predicted/observed: R^2 = 0.94 [31]	Sampling [31]	[31]
Fatty acids	ISM/Image recognition	DHA/cell diameter: R^2 = 0.98 (calibration)	PBR in-situ probe	[34]
Protein	Chlorophyll fluorometry	Protein/Chl a fluorescence: r = 0.92	Fiber probe in PBR bypass	[35]
Carotenoids (C)	VIS/NIR spectrum	Predicted/observed C: r = 0.96	Fiber in sample	[32]

2.1. Biomass Concentration

Biomass concentration (BC, mostly expressed as dry weight concentration, DWC) is the basic biological variable in any microbial cultivation. Measurement of optical density (OD) is the most widely employed proxy for the BC. By far the most implementations of on-line sensors other than standard turbidity sensors measure (or estimate) the BC. To use optical density as a proxy for BC reliably and accurately, a choice must to be made concerning the suitable wavelength and the proper conditions for the OD/DWC calibration, and some assumptions have to be—at least approximately—fulfilled in order for this proxy to work: biomass composition should not change significantly during measurement or within the measurement range; for calibration, cells from different growth phases should be preferably used; ideally, calibration should change along the cultivation path reflecting thus changes in the optical properties of the biomass due to the changing cell size (scattering method) or changing composition.

Griffiths et al. [60] recommend selection of a wavelength with minimal pigment absorption, e.g., 750 nm, and calibration in the middle of or across the entire growth cycle. For routine measurements, optical density at 750 nm (OD_{750}) is widely used to monitor algal growth [61] as it avoids the absorption of light by cellular pigments (chlorophyll and carotenoids) and is treated as a pure light scattering measurement. The major drawback is that light scattering is an aggregate variable of cell size, density, opacity and granularity which is difficult to take apart and may also be influenced by the presence of bacteria and inorganic solids.

Nielsen and Hansen [21] argue that OD measured within the absorption range of chlorophyll provides more specificity for measuring microalgal biomass when other particles are present but, on the other hand, measurements would be sensitive to changes in the chlorophyll content of the microalgal biomass and, thus, measuring OD within the absorption range of chlorophyll has both advantages and disadvantages compared to using the 750 nm wavelength. Any of the investigated wavelengths of 550, 665 and 750 nm could serve as a robust proxy for the biomass concentration. In another study [62], the authors conclude that correlation between measurements at 480, 510, 630, 647, 650, 664, and 750 nm is rather high, with Pearson coefficients $\rho = 0.92–0.97$, so the wavelength choice between those investigated does not seem to be very important.

2.2. Monitoring BC Using Direct Sensors without Complex Signal Evaluation

Barbosa et al. [19] designed a low-cost multi-wavelength (400, 850, 940 nm) absorbance sensor for real-time monitoring of the OD in microalgal cultures in both closed and open systems. Light from three LEDs was transmitted through a glass tube placed in a PBR bypass, and linear correlations between ODs measured at individual wavelengths and biomass DWC measured by gravimetry were found in the range 40–800 $mg \cdot L^{-1}$ DWC. The same procedure was used for correlating DWC with OD measured at five wavelengths (400, 680, 750, 850, and 940 nm) in a spectrophotometer. Correlation coefficients (R^2) for individual correlations were in the range 0.81 to 0.96. No OD combination at different wavelengths for a multi-wavelength correlation was attempted.

Nguyen and Rittmann [16] suggested measuring BC by the far red TSD-10 turbidity sensor employed commercially in washing machines, OD_{730} was linear from 0.5 to 4.5 rel. AU in a *Synechocystis* culture. The sensor was calibrated using a spectrophotometer at 730 nm and tested as a BC sensor installed in a bypass in a 2.5-L working volume flat panel PBR operated as a turbidostat. With automatic harvesting, the turbidostat could maintain stable BCs in step-down and step-up experiments.

Sarrafzadeh et al. [15] compared nine techniques for measuring the cell concentration using four different microalgae cultivated in a closed PBR, using DWC as the reference. The investigated methods comprise the manual cell count, microscopic automated cell count (benchtop Countess, Invitrogen), OD_{680} measured in a spectrophotometer, dielectric permittivity (Evo 200, Fogale Nanotech, Nimes, France), NIR OD (nephelometry 90° scatter, NIR-980 probe, Evo 200), oxygen production rate (OPR), RGB analysis of images captured

off-line [27], flow cytometry with fluorescence and forward and side scatter. All methods apart from DWC, manual cell count and OPR require only several minutes to produce results. RGB, OD (NIR) and the dielectric permittivity method show potential for on-line application, the latter two being carried out with commercial sensors Evo 200. No details concerning the measurement setup with Evo 200 (NIR-OD, permittivity) are described.

Thoré et al. [18] monitored BC of three species (*Chloromonas typhlos*, *Microchloropsis gaditana* and *Porphyridium purpureum*) in closed, pilot-scale PBRs on-line and in real time using a flow-through nephelometer and related nephelometric turbidity (in NTU) to DWC and optical density at four wavelengths ranging from 435 to 720 nm. The resulting relationships between turbidity (NTU) and DWC and between DWC and OD_{720} were nonlinear, with R^2 = 0.88–0.93 and R^2 = 0.88–0.92, respectively, differing between species.

Jia et al. [20] used a multi-wavelength OD sensor measuring OD at 650, 680 and 780 nm in an on-line, real-time mode, with light generated by laser diodes placed in a flow cell in a bypass, both in a closed PBR and in an open raceway. Measurements at 650 and 680 nm correlated with the chlorophyll content, while measurement at 780 nm estimated the suspension turbidity. For BC estimation, only OD_{780} was used, and the other wavelengths were used only for monitoring the change in growth phases by computing ratios OD_{650}/OD_{780} and OD_{685}/OD_{780}.

Salmi et al. [30] estimated BC in an off-line mode by a commercial hyperspectral camera for imaging suspension samples (24- or 96-well plates) of five microalgal strains in the exponential growth phase. From the transmittance spectra, ratios of transmittance at 751/676 nm wavelengths (width 7 nm) were successfully correlated with the BC expressed as DWC or cell count (measured by an electronic cell counter or single-channel fluorometry) in a BC range differing by the strain.

2.3. Monitoring Biomass Concentration Using Complex Evaluation

Most monitoring methods in this section use fluorescence as the measured process signal. Other signals include optical density, reflectance, RGB and a combination of pO_2, pH, gas flow (air, CO_2) and solar radiation. "Complex evaluation" means that signals of hardware sensors are processed by software sensors in the form of observers, chemometric models, or artificial neural networks (ANN), all described in Section 4.

2.3.1. Methods Based on Optical Density Measurement

Flores et al. [17] use a sensor like that of Nguyen and Rittmann [16] which is employed for measuring the OD_{560} of a suspension of mixotrophically cultivated *Spirulina* flowing inside a borosilicate tube, calibrated with biomass DWC. A flat-panel closed PBR was used for cultivation. The turbidity signal was processed by a robust nonlinear observer to deliver an estimate of the microalgal biomass concentration and of the substrate (glucose).

2.3.2. Methods Based on Fluorescence Measurement

Chemometric models for fucoxanthin content and biomass concentration of *T. lutea* and *P. tricornutum* were developed by Gao et al. [63], using 2D fluorescence excitation-emission matrices (EEM) obtained by off-line measurements in a cuvette as the only inputs. Biomass models predicted the BC with R^2 between 0.93 and 0.96.

In another study, reflection spectra (not used) and fluorescence were acquired simultaneously by a "Y" optical fiber reflecting probe with seven fibers (6× excitation light + 1× fluorescence readout) immersed in the sample [39]. Acquired fluorescence signals were correlated using ANN or Genetic-Algorithm-ANN with a hemocytometer count. In more detail, the study is described in Section 4.3.

Sa et al. [48] acquired fluorescence spectra of a *N. oceanica* suspension in an off-line setup (spectrophotometer cuvette), and the resulting EEMs were correlated using a chemometric model (N-partial least squares (PLS), multiway PLS, in MATLAB) with the cell concentration, chlorophyll a, and total, saturated, and unsaturated fatty acids. Inner filter effects were compensated for. Cell concentration was estimated from EEMs with

R^2 = 0.66–0.97. This method could be potentially converted to an on-line implementation using a submerged fiber fluorescence probe.

Fluorescence EEMs were measured in samples drawn from a cultivation of *Dunaliella salina* by Sa et al. [41]. Samples were measured by an immersed optical fiber probe without any sample treatment, excitation 250–690 nm, emission 260–700 nm. Principal component analysis (PCA)/PLS was used to design a chemometric model using fluorescence PCs as inputs and cell concentration, cell viability and nitrate concentration as outputs. Converting to an on-line application is suggested (but not performed) by coupling the fiber probe to the PBR which would provide the biomass estimation with about a 5 min delay.

Shin et al. [38] designed a hand-held fluorometer using three different wavelengths of LEDs (385, 448, 590 nm) to stimulate fluorescence emission at 645 and 680 nm. The resulting fluorescence pattern is processed using a chemometric model built using PLS regression for selective BC estimation of *C. vulgaris* and *Spirulina* in a mixed sample. Measurements were carried out in a micro-vial and required sampling, but the principle could be easily adapted to an on-line implementation. Devices estimating microalgal and cyanobacterial BCs based on this approach were designed by this author group previously, using a PDMS microfluidic chip and the three LEDs with wavelengths 385, 448 and 590 nm but without the chemometric model [37]. The initial version used excitation at only one wavelength (448 nm) [36] and the authors showed that the non-microalgal turbidity of the medium did not influence the measurement.

Sa et al. [40] use an optical fiber bundle probe in a stirred beaker containing sample from a *D. salina* cultivation measuring 2D fluorescence spectra (EEMs), with excitation at 250–690 nm and emission at 260–700 nm. PCA and PLS chemometric models were designed to estimate cell count and cell viability (percentage of cell disruption) using as inputs the emission intensity in three fluorescence regions identified in the 2D fluorescence spectra matrices.

Perin et al. [58] estimated biomass by measuring chlorophyll fluorescence of microalgae grown in a microfluidic device with microwells with a FluorCam FC 800, a system for combined multispectral and kinetic fluorescence imaging consisting of LED panels and a CCD camera where the measured object is placed inside the measurement box. This setup is suitable only for microwells or a similar setup, not for a standard photobioreactor.

On-line fluorescence measurements were performed by Karakach et al. [35] in a *Scenedesmus* cultivation using a submersible probe equipped with a USB4000 spectrometer (Ocean Optics, Dunedin, FL, USA) placed in an external recirculation loop of a closed PBR. In parallel, culture turbidity was measured with a commercial turbidity sensor. Acquired fluorescence spectra were analyzed using linear regression for DWC and cell count and using principal component regression (PCR) for the protein concentration. Chemometric models for estimations of the BC (as DWC and cell number concentration) and of the protein concentration based on chlorophyll-related culture fluorescence were thus designed. When used in real-time environment, the DWC and cell number concentration estimations had an acceptable accuracy (r = 0.95 and 0.92, respectively) using the linear regression, estimation of protein concentration required the more complex PCR model which attained r = 0.8.

2.3.3. Methods Based on Color Measurement

Benavides et al. [26] developed a low-cost RGB turbidity sensor based on the commercial ColorPAL module that measures the intensity of light generated by three LEDs (580, 540 and 450 nm) and absorbed in a polystyrene flow-through cuvette. The measured red, green, and blue signals were converted to luminance and absorbance in an Arduino microcomputer and employed to compute the BC using a calibration based on absorbance measured off-line at 680 nm in a spectrophotometer which in turn was calibrated using gravimetry. Results obtained with the RGB sensor placed in a bypass of a closed flat panel PBR in a culture of *D. tertiolecta* were compared both to the off-line determination with a spectrophotometer as well as to turbidity measured by the commercial Optek ASD19-N

probe. The accuracy and precision of the RGB sensor, spectrophotometer and the Optek probe were comparable, with R^2 = 0.998 for the correlation DWC vs. the absorbance measured by the RGB sensor. For control purposes, BC estimated by the RGB sensor were employed in a Droop model-based extended Luenberger observer to estimate the nitrate concentration and the intracellular quota in a continuous cultivation of *D. tertiolecta*.

Murphy et al. [28] quantified BC in biofilms and in suspended microalgal cultures using wideband multispectral imaging of reflected and backscattered light. Culture images were taken with a simple RGB camera, and the resulting images analyzed for the RGB components. The measurement both in biofilms and in the suspended culture was carried out in off-line mode but the suspended culture measurement could be adapted for an on-line implementation. In both biofilms and suspended cultures, correlations between the areal BC and separate intensities of the R, G and B components were found, with R^2 = 0.97–0.99. This method was further developed for use in suspended cultures [29] where the backscattered light is measured and its RGB intensities analyzed to correlate with the BC, invasion of the microalgal culture by cyanobacteria and with the decrease in the photosynthetic yield. The average error between the actual and predicted BC based on measured RGB values was 14 to 22% depending on the microalgal strain.

2.3.4. Methods Based on Reflectance Measurement

Lopez-Exposito et al. [23] used a focused beam reflectance probe (FBRM) [64] which measures the chord length distribution (CLD), to represent the particle length distribution and to estimate the particle size. Data were acquired in samples drawn from a PBR and were processed by a perceptron (ANN) that correlated the measured CLD with the BC obtained by gravimetry, attaining a correlation coefficient of R^2 = 0.92. In a further development of this method [24], reflectance data obtained by the same rotating laser probe and BC data of *C. sorokiniana* in samples drawn from a PBR were processed with a support vector regression and a random forest regression model, both methods of machine learning, attaining the respective accuracy of R^2 = 0.87 and 0.81 in the biomass dry weight estimation. As in [23], flocculant was used in parallel to check the influence of the aggregation state and turbidity on the biomass estimation result.

2.4. Mixed Culture Discrimination

Franco et al. [65] identified contamination of single-strain microalgal cultivations by other microalgal species using measurements of light absorption in each separate microalgal culture at 31 points between 400 and 700 nm in bandwidths of 10 nm on five consecutive days. Four microalgal strains were used: *Nostoc*, *Scenedesmus*, *Spirulina* and *Chlorella*. Spectral signatures obtained in this way were used to train an ANN that could then identify pure individual species with an accuracy >98.7%. The trained ANN could differentiate between monoalgal and mixed algal cultures and of identifying contamination of a single-strain culture by another species if the addition was higher than 10%. When used as a preliminary test to a microscopic examination, this method would speed up the identification of a culture contamination.

2.5. Cell Count (Cell Number Concentration)

To determine the cell number concentration, Kiss and Nemeth measured permittivity on-line [56] using a commercial capacitance-based viable cell count sensor (Incyte, Hamilton Comp., Reno, NV, USA) equipped with the corresponding signal transmitter and controller (Fogale Nanotech, France). The signal correlated well (R^2 = 0.999 for *Nannochloropsis* sp., R^2 = 0.992 for *C. vulgaris*) with the OD_{560} measured in a spectrophotometer during calibration. In a cultivation in a closed PBR, however, the biomass concentration data scatter computed from the permittivity data was significantly higher than that computed from the off-line OD_{560}, for unknown reasons. The drawback of the permittivity sensor is the relatively low signal-to-noise ratio.

Zhou et al. [25] correlated cell counts of three microalgal species (two eukaryotes and one cyanobacteria strain) with the hyperspectral reflectance (HR) and with the hyperspectral extinction coefficient (EC) in the NIR region (695–750 nm). Cell counts were in the range of 2.5×10^5 to 1.8×10^8 cells·mL^{-1}, and the R^2 of the linear dependence cell count vs. HR or EC in this range was over 0.99. Measurements were performed in a 1200 mL open, flat, glass container. Method development was aimed at its use in remote sensing, but the method could also be used as a rapid in-situ measurement.

A chemometric model for monitoring cell count and viability in a *D. salina* cultivation was developed by Sa et al. [40] based on a 2D fluorescence measurement. Fluorescence was measured in a drawn sample using an immersed fiber probe. The setup could be adapted for an on-line measurement.

2.6. Cell Morphology

The single-cell size distribution of the heterotrophically cultivated microalga *Crypthecodinium cohnii* was monitored in a closed bioreactor using an in-situ microscope (SOPAT GmbH) coupled with an automated image analysis system based on an ANN trained with user-annotated images [33]. The cell size distribution was found to correlate with the cell content of docosahexaenoic acid (DHA), which can be thus indirectly monitored without sampling and cell disruption. In a similar fashion, DHA was monitored in a heterotrophic culture of *C. cohnii* in a closed bioreactor using the ISM directly in the culture broth and the 3D digital holographic microscopy on a microscope slide, based on the cell size and width of the size distribution [34].

2.7. Culture Health Monitoring, Contamination

Reflectivity spectral data were collected on-line by Reichardt et al. [22] in four open raceway ponds cultivating *N. oceanica* and *C. vulgaris* using multi-channel spectrometers, equipped with downward-looking fibers angled at 30° from normal, one at each pond, for monitoring the reflected light, and one upward-looking fiber equipped with a diffusing optics (180°) for monitoring the downwelling light. Reflectance spectra were analyzed by a model described in [66]. Natural diatom invasion of *N. oceanica* and grazing of *Chlorella* were investigated. Further invasion detection in other microalgal cultures was investigated under laboratory conditions. Both grazers and competitors could be successfully detected.

A grazer (*Oxyrrhis marina*) was detected in open pond *Dunaliella* cultures using FTIR-based off-line spectra acquisition in combination with a PLS regression chemometric model [67]. The 1363 cm^{-1} wavenumber could be used as a potential marker for the grazer as early as 72 h prior to the culture crash. Although carried out in the off-line mode, this method has a potential for an on-line implementation.

In another study, early detection of the predation by *O. marina* or *Euplotes sp.* in a shake flask culture of *D. tertiolecta* was achieved by monitoring non-photochemical quenching (NPQ) by means of PAM fluorometry [47]. NPQ levels were found to decrease significantly 24 to 48 h prior to the culture crash.

Sauer et al. [59] exploited the production of volatile gases by a microalgae culture attacked by grazers to continuously monitor the culture for signs of contamination. Nitrogen-containing gases including ammonia and pyrroline were found to be reliable indicators of grazing. Real-time measurements were performed using time-of-flight chemical ionization mass spectrometry on cyanobacterial monocultures of *S. elongatus* before and after the addition of the grazer *Tetrahymena*. Presence of the grazer could be detected as early as 18 h after grazer addition, with the range of 18–67 h depending on the detected gas (NH_3, monoterpenes, pyrroline), on average twice as fast as by microscopy and three times as fast as by continuous fluorescence measurement.

2.8. Species Identification and Classification

Only off-line methods for species identification can be found. Xu et al. [68] used hyperspectral microscopic imaging with machine learning. Deglint et al. [69] processed

multispectral microscopic images acquired off-line, using absorption at 465–660 nm (seven wavelengths) and fluorescence with excitation at 385 and 405 nm, with signals processed by an ANN.

2.9. Photosynthetic Efficiency, Quantum Yield

During cultivation, the photosynthetic activity of microalgae changes and is a suitable indicator of growth [45]. Most measurements of the photosynthetic efficiency or quantum yield (PQY) by PAM have been carried out in dark-adapted samples after incubating a drawn culture sample in dark for about 5–10 min [70]. The dark adaption can also be carried out in an on-line setup [42]. On-line measurements in light-adapted samples are rather infrequent (see Section 3.3 in [10]). Recent examples are the identification of failures of CO_2 supply by the in-situ, on-line monitoring of the PQY by a PAM fluorometer together with monitoring differences in dissolved oxygen concentration in two locations along an open outdoor TLC photobioreactor [45]; further estimation of biomass productivity in *Chlorella* cultivated in a TLC open outdoor PBR by simultaneous on-line measurement of irradiance and quantum yield by a PAR sensor and a PAM fluorometer with fiber optics both submerged in the culture [44]; and identification of the lipid accumulation onset in *Nannochloropsis oceanica* cultured in closed Roux bottles by measuring in situ the effective PSII quantum yield ($\Delta F'/F_m'$) and finding also a pronounced correlation between the effective quantum yield and the lipid content [46].

2.10. Biomass Composition

2.10.1. Lipids

Bouillaud et al. [55] performed on-line, non-invasive total lipid measurements in a closed PBR circulation loop using a compact benchtop NMR spectrometer. The NMR signal correlated well ($R^2 > 0.99$) with the FAME total lipid analysis by GC-FID. The water signal could be selectively removed using a WATERGATE pulse sequence. Spectra were acquired for 1 h. Lipid concentrations in the culture as measured by NMR were in the range 25–480 $mg \cdot L^{-1}$. A proof-of-concept for this method was carried out previously by the same group [53] with a desktop benchtop NMR for on-line, non-invasive lipid detection in microalgae in a PBR bypass, with preliminary work to optimize the water peak suppression. NMR spectra of three cultures of *N. gaditana* with different lipid content were acquired showing distinct lipid concentration dependent peaks. The employed instrument and method were shown to be reasonably sensitive to measure the evolution of lipid concentration.

A low field NMR device was used by Wang et al. [52] to acquire signals from a cultivation of *C. protothecoides* in an on-line setup, where samples from a PBR were automatically drawn with a pump, mixed with $MnCl_2$ to suppress the water signal and pumped into the measuring chamber of the NMR. Method feasibility was tested by using glyceryl trioleate as standard. Calibration for the quantitative lipid monitoring was carried out using the extraction and GC-MS methods for lipids.

Various nonlinear estimators (Extended Kalman Filter, EKF; Unscented Kalman Filter, UKF; Particle Filter, PF) used for on-line estimation of the lipid concentration in mixotrophic cultivations of *C. protothecoides* are compared by Yoo et al. [71]. As estimator inputs, on-line turbidity calibrated with the biomass DWC data and off-line glucose measurements were employed and light intensity and flow rates of glucose and nitrogen containing feed were varied to test the estimator ability to track the lipid concentration determined by Nile Red fluorospectrometry. The UKF lipid estimator developed here was further used in [72] for the model-based real-time optimization of a *C. protothecoides* cultivation using the model predictive control (MPC) method, with on-line turbidity data calibrated with biomass DWC and off-line glucose data used as process inputs for the UKF estimating the lipid concentrations and the MPC calculating process control inputs (light intensity, glucose and glycine feed rate) for the optimal cultivation course.

Carneiro et al. [46] could identify the onset of lipid accumulation in *N. oceanica* cultured in closed Roux bottles by in-situ monitoring of chlorophyll a fluorescence using a PAM fluorometer. The effective quantum yield of PSII ($\Delta F'/F_m'$) could be successfully correlated with the cell lipid content as %DWC.

There are reports of several methods for lipid estimation carried out in an off-line mode that could be adapted for on-line monitoring. Among them is the work of Sa et al. [48,49], who developed chemometric models for indirect measurement of cell count, chlorophyll and fatty acids based on fluorescence measured off-line in a cuvette. An on-line adaptation would be possible if a fiber probe were used, as in other publications from this group [40,41,50].

An adaptation for on-line should be possible for the monitoring described in [48], in which a chemometric model was developed using as inputs the fluorescence EEM with fluorescence acquired from samples placed in a cuvette of an external spectrophotometer, in excitation ranges 250 to 790 nm for excitation and 260 to 800 nm for emission. The model predicts five cultivation parameters: cell count, chlorophyll, and fatty acids as total, saturated and unsaturated. Total fatty acids were estimated with $R^2 = 0.78$. The concentration of eicosapentaenoic acid was monitored with a similar approach (a chemometric model using EEM as the sole inputs) and measurement setup in [49].

The method applied by Li et al. in [31] required sampling and acquiring transmissivity images in the NIR region (1000–1350 nm) using a platform with a hyperspectral imaging camera. Five different chemometric models were tested and the CARS-MLR model using spectra smoothed by a Savitzky–Golay filter was selected for the estimation, with prediction performance of $R^2 = 0.94$ against the gravimetric lipid analysis.

A label-free dielectrophoresis-based microfluidic sorting platform that can separate microalgal cells into six outlets based on their intracellular lipid content was designed by Han et al. [57]. This effect could be used to monitor and quantify the distribution of intracellular lipid level of a given microalgal cell population as a high throughput or an on-line method.

The neutral lipid [73] and lipid and carbohydrate [74] contents in microalgal cells of *I. galbana* under nitrogen stress in a continuous culture were estimated by adaptive interval observers based on simple process models with Monod and Droop kinetics. As observer inputs, measurements of biomass by an optical particle counter in 1 h intervals and measurements of residual nitrate by an auto-analyzer every 2 h were used.

2.10.2. Carbohydrates

In a further development of the lipid estimator in [73], neutral lipid and carbohydrate contents in microalgal cells of *I. galbana* under nitrogen stress in a continuous culture were estimated by adaptive interval observers based on simple process models with Monod and Droop kinetics with measurements of biomass by an optical particle counter and residual nitrate as observer inputs [74].

2.10.3. Pigments

Predictive chemometric models were developed for chlorophyll content (a, b and total) and carotenoid content (total carotenoids and four specific carotenoids) in *D. salina* cultivated in closed outdoor and indoor photobioreactors [50]. As input data, the models used 2D fluorescence (EEM) measured with a fiber probe in samples and, for carotenoids, climatic data (temperature, precipitation, sunlight, PAR radiation, cloud fraction, irradiance).

A chemometric model was developed using as inputs the fluorescence EEM in a *N. oceanica* cultivation [48]. Fluorescence EEMs were acquired from samples placed in a cuvette of an external spectrophotometer. The model predicts chlorophyll concentration with $R^2 = 0.75$–0.85.

Another chemometric model, mentioned already in Section 2.3.2, was developed for estimation of fucoxanthin content and biomass concentration [63]. Fucoxanthin models predicted its contents with R^2 between 0.63 and 0.77.

Shao et al. [32] estimated carotenoid concentration in *Spirulina* by using VIS/NIR transmission spectra 346–1038 nm by 0.3 nm acquired with a USB4000 (Ocean Optics, Dunedin, FL, USA) fiber optic probe immersed in a test tube and a halogen light source. A chemometric model was developed using the LW-PLS, UVE-PLS and SPA-PLS method, with the latter giving the best results.

All four of these methods of pigment estimation could probably be adapted for on-line measurement by a suitable installation of a fiber fluorescence probe in some type of PBR bypass.

2.10.4. Proteins

On-line fluorescence measurements were performed in a *Scenedesmus* cultivation using a submersible probe equipped with a USB4000 spectrometer (Ocean Insights, Tokyo, Japan) placed in an external recirculation loop of a closed PBR [35]. In parallel, culture turbidity was measured with a commercial turbidity sensor. Acquired fluorescence spectra were analyzed using linear regression for DWC and cell number concentration and using PCR (principal components regression) for the protein concentration. Chemometric models for estimations of the cell density (DWC, cell number concentration) and of the protein concentration based on chlorophyll-related culture fluorescence were thus designed. When used in real-time environment, the DWC and cell count estimations have shown an acceptable accuracy (r = 0.95 and 0.92, resp.) using the linear regression, estimation of protein concentration required the more complex PCR model which attained r = 0.8.

3. High-Throughput Methods for Monitoring of Biological Variables

Although this review is focused on on-line sensors, monitoring of biological variables in microalgal cultivations by using off-line methods capable of mass processing of drawn samples in a fast and reliable manner (hence the name "high-throughput methods") deserves a short mention here because some of these methods, mostly those using some type of optical sensing, could be adapted for on-line, in-situ use.

High-throughput methods present a special category of rapid analytical methods used for mass analyses not only in microalgal cultivations but in microbial cultivations in general. They are optimized mostly for parallel analysis of many cultivation samples or small volume cultivations performed in well plates. With microalgae, a typical application is screening for neutral lipids using fluorescent stains, mostly Nile Red or BODIPY [75–77]. The fluorescence-based staining methods fight a problem of the uniform dye penetration into cells which must be facilitated by different solvents [76–78]. A fast method toolbox has been presented by Palmer et al. [79] for screening for phycobiliproteins, chlorophylls, carotenoids, proteins, carbohydrates, and lipids using simple colorimetric methods with the purpose of strain selection and optimization. An estimation of carotenoid concentration in *Spirulina* using VIS/NIR transmission spectra was investigated in [32] with spectra obtained by an Ocean Insights (USA) fiber optic probe immersed in a test tube. This method could be also adapted for on-line use.

4. Computer-Aided Monitoring and Software Sensors

In all biotechnological processes, one can find variables that cannot be measured directly in real time without substantial effort, human or instrumental, because of a lack of suitable sensors. Available sensors are too inaccurate, unstable, expensive, or there are no sensors available for the variable at all. Then, process control theory allows us to use so-called state observers that use signals of available sensors and convert them, through help of a mathematical model of the process, into indirect measurement of the desired variable. Pattern recognition in the form of ANNs or sophisticated multiple regression methods as used in chemometric models is another way to the same end, obtaining values of process variables that cannot be measured directly. These estimators are usually called "software sensors" to stress that they provide values of process variables as if they were provided by physical sensors, to be used for monitoring and control

purposes. The first estimator type, the model-driven estimator, uses mostly mass and energy balance process models and kinetic models, implemented in observers (Luenberger observer), adaptive observers, interval observers or statistical filters of the Kalman–Bucy type [80]. In model-driven estimators, quality of the software sensor depends decisively on the quality of the underlying mathematical model of the process. The second estimator type, the data-driven estimator, is represented by some type of multivariable correlations between measured and target variables, as in chemometric models [4,81,82], or ANNs [83] performing pattern recognition between measured and target variables. Combinations of all these approaches are widely used in hybrid models [80]. In a certain sense, practically all modern sensors implementing complex sensing methods are software sensors because the primary signals must be less or more processed by some software to provide the value of the desired process variable, either in real time or with some delay. A good example is NMR spectroscopy, which requires formidable computing power implemented in the hardware instrument to perform multistep data processing and deliver a single current value of lipid concentration [55]. Sometimes it is rather difficult to decide if the indirect measurement should be classified as a software sensor, e.g., a nonlinear regression model—as in chemometrics—using as input absorption measured at multiple wavelengths. However, this question is much more academic than practical.

Various process variables are estimated in the following overview of software sensors used in microalgal cultivations: biomass, cell count, cell viability, concentration of lipids, carbohydrates, glucose, sulfur, nitrate, chlorophylls a and b, carotenoids, total fatty acids and EPA, and contamination of a single-strain cultivation by other microalgal strains. Process variables measured with a hardware sensor or method either on-line or off-line employed as estimator inputs are cell count by a particle counter, concentration of nitrate and glucose, temperature, OD, output flow O_2, output flow CO_2, turbidity, air injection flow, CO_2 injection flow, irradiance, fluorescence spectra, hyperspectral OD, reflectance and 2D fluorescence in the form of EEMs.

Most model-based estimators belong to one of two categories. In the first category there are observers that correct the values generated by the process model using some readily measurable process value with a constant gain selected during the observer design. The constant gain can also be continuously adapted by some available technique [84], and interval observers can limit the state trajectory based on the known intervals of uncertain model parameters [73]. The second category are variants of the Kalman filter which alters the correction gain in each iteration recursively using the comparison between noisy state estimations and noisy measurements, using covariance matrices of the system and measurement noises whose selection is critical for the proper functioning of the filter [85].

The most often encountered process variable estimated by software sensors in microalgal cultivations is the biomass concentration using several physical measurement signals: turbidity, outlet gas composition, pO_2, pH, irradiation intensity, fluorescence and reflectance. It is followed by the lipid content based on cell count, nitrate, turbidity, glucose (off-line), IR spectra (off-line with ATR-FTIR) and 2D fluorometry spectra (off-line, but adaptable to on-line). Further process variables estimated by means of software sensors include carbohydrates and proteins, intracellular nitrate quota, substrate (glucose), sulfur, cell count, cell viability, concentration of chlorophyll a and b, carotenoids, total fatty acids and EPA. Software sensors measuring biological parameters in microalgal cultivations reported in recent years are summarized in Table 3.

The accuracy of the individual software sensors reviewed here and listed in Table 3, together with the estimated biological variable, software sensor type and measurement conditions and limitations is shown and compared in Table 4. Numerically expressed accuracy is reported only with chemometric models. Results produced by observers, Kalman filters and ANNs are, as a rule, reported in original publications only in graphical form depicting the comparison of measured and estimated data during the cultivation. That reflects the fact that these estimators work dynamically, providing step-by-step estimation of process state variables based on measured process outputs. Several software sensors

listed in Table 4 are implemented on-line, some work with experimental data supplied off-line but could be adapted to a true on-line implementation, and some are implemented in an off-line mode.

4.1. Observers

Adaptive interval observers based on simple process models with Monod and Droop kinetics were developed that can estimate the neutral lipid [73] and lipid and carbohydrate [74] contents in microalgal cells of *I. galbana* under nitrogen stress in a continuous culture based on measurements of biomass by an optical particle counter and residual nitrate.

Two software sensors are proposed in the form of extended Luenberger observers, based on the Droop model [84]. Parameters of the Droop model were identified using on-line measurements of temperature, optical density and extracellular nitrate concentration. The first observer estimates the substrate (nitrogen) concentration in medium and the internal nitrogen quota from the measurement of biomass through optical density, the second observer estimates the intracellular nitrogen quota from the measurement of biomass and the extracellular nitrogen concentration.

As described in Section 2.3.1 in more detail, a robust nonlinear observer processing a turbidity signal was used to estimate the microalgal biomass and glucose concentration [17].

Table 3. Software sensors monitoring biologically important variables in microalgal cultivations.

Variables Monitored by the Software Sensor	Input Variables (On-Line When Not Otherwise Stated)	Software Sensor Type	References
Lipids Carbohydrates	Particle counter Nitrate (assumed measured on-line)	Adaptive interval observer	[73,74]
1. Extracellular nitrate, intracellular nitrate quota 2. Intracellular nitrate quota	1. OD (biomass) 2. OD (biomass), extracellular nitrate	Luenberger observer	[84]
Biomass Glucose	Turbidity	Robust nonlinear observer	[17]
Biomass Sulfur	Outlet gas (O_2, CO_2) by MS	EKF	[86]
Lipids	Turbidity Glucose (off-line)	EKF, UKF, PF	[71,72]
Biomass	pO_2 pH, air flow CO_2 flow solar radiation	EKF	[85]
Cell count	Fluorescence spectrum (off-line)	ANN	[39]
Contamination	Multispectral absorption (off-line)	ANN	[65]
Biomass	Reflectance (off-line)	ANN	[23]
Biomass	Reflectance (off-line)	SVR, RF regression	[24]
Protein, lipids, carbohydrates	IR spectrum (ATR-FTIR) off-line	Chemometrics	[87]
Cell count Cell viability Nitrate concentration Chlorophyll a, b concentration Carotenoids Total fatty acids EPA	2D fluorometry (EEM) (off-line, adaptable to on-line)	Chemometrics	[40,41,48–50]
Biomass (X) Fucoxanthin (Fx)	2D fluorometry (EEM)	Chemometrics	[63]
Carotenoids	Transmission spectrum	Chemometrics	[32]

Table 4. Accuracy of software sensor methods where numerically available. In most cases, only graphs comparing the time course of variables' estimation vs. their measurement are shown in the original article (denoted here as "graphic comparison").

Biological Variable	SW Sensor Type	Method Accuracy	Limitations/Conditions	Reference
Lipids	Adaptive interval observer	Graphic comparison 35 days	Tested with experimental data, adaptable to on-line	[74]
Carbohydrates		Graphic comparison 35 days		[73]
Extracellular nitrate, intracellular nitrate quota Intracellular nitrate quota	Luenberger observer	Graphic comparison 4–6 days	Tested with experimental data, adaptable to on-line	[84]
Biomass Glucose	Robust nonlinear observer	Graphic comparison 18 days	On-line implementation	[17]
Biomass Sulfur	EKF	Graphic comparison 8–10 days	On-line implementation	[86]
Lipids	EKF, UKF, PF	Graphic comparison 300 h	On-line implementation	[72]
		Graphic comparison 300 h		[71]
Biomass	EKF	Graphic comparison within 1 day	On-line implementation	[85]
Cell count	ANN	Graphic comparison 10 days	Adaptable to on-line	[39]
Contamination	ANN	Identification of 4 pure species Accuracy > 98.7%	Measured in samples	[65]
Biomass (X)	ANN	Predicted/observed X: R^2 = 0.92	Measured in samples	[23]
Biomass (X)	SV regression	Predicted/observed X: R^2 = 0.87	Measured in samples	[24]
	RF regression	Predicted/observed X: R^2 = 0.81		
Protein (P)	Chemometric models	Predicted/observed P: R^2 = 0.88/0.92/0.85	Freeze-dried samples	[87]
Lipids (L)		Predicted/observed L: R^2 = 0.82/0.90/0.77		
Carbohydrates(C)		Predicted/observed C: R^2 = 0.65/0.77/0.63		
Ratio carbohydrates/proteins		Predicted/observed C/P: R^2 = 0.84		
Cell count (CC)		Predicted/observed CC: R^2 = 0.66–0.97	Adaptable to on-line	CC, CV: [40] CC, CV, N: [41] CC, Chl, TFA: [48] EPA: [49] Chl, C: [50]
Cell viability (CV)		Predicted/observed CV: R^2 = 0.69		
Nitrate concentration (N)		Predicted/observed N: R^2 = 0.80		
Chlorophyll a, b concn. (Chl)		Predicted/observed Chl: R^2 = 0.75–0.85		
Carotenoids (C)		Predicted/observed C: R^2 = 0.72–0.89		
Total fatty acids (TFA)		Predicted/observed TFA: R^2 = 0.78		
EPA fraction in TAG		Predicted/observed EPA: R^2 = 0.87		
Biomass (X) Fucoxanthin (Fx)	Chemometric model	Validation X: R^2 = 0.93–0.96 Validation Fx: R^2 = 0.63–0.77	Measured in samples	[63]
Carotenoids (C)	Chemometric model	Predicted/observed C: r = 0.96	Measured in samples	[32]

4.2. Kalman Filters

An extended Kalman filter estimates the biomass concentration and the extracellular and intracellular sulfur concentration in a closed *C. reinhardtii* cultivation producing hydrogen [86]. A Droop model with the internal sulfur quota is used as a basis, describing the microalga growth under light attenuation and sulfur-deprived conditions. The observer uses on-line measurements of the outlet gas composition, oxygen and carbon dioxide, by a mass spectrometer.

The applicability of various nonlinear estimators for on-line estimation of the lipid concentration in a closed microalgal cultivation system (*C. protothecoides*, mixotrophic) was examined and compared in [71]. All estimators were based on the cultivation model described in [88] from which the lipid consumption rate term was omitted. On-line turbidity (calibrated with the DWC data) and off-line glucose measurements were employed as estimator inputs and light intensity and flow rates of glucose and nitrogen containing feed were varied to test the estimator ability to track the lipid concentration determined by Nile Red fluorescence. Results show that the EKF is not suitable in this case, but the UKF and PF displayed satisfactory performances. The UKF lipid estimator developed here was further used in [72] for the model-based real-time optimization of a *C. protothecoides* cultivation in a closed bioreactor using the model predictive control (MPC) method. As in the previous case, on-line turbidity data calibrated with DWC with a 5 s sampling rate and off-line glucose data in 1 h intervals were used as process inputs for the UKF estimating the lipid concentrations, and all three variables were fed into the MPC to calculate process control inputs (light intensity, glucose and glycine feed rate) for the optimal cultivation course.

A state estimator with the EKF was developed to estimate biomass concentration in an outdoor raceway cultivating *Scenedesmus* [85], based on a dynamic model of the process [89]. As inputs for the EKF, on-line measured values of dissolved oxygen, pH, injected flows of air and CO_2 and solar radiation were used. The crucial point is the appropriate determination of covariance matrices for the EKF for which complete data of the entire cultivation season are necessary.

4.3. Machine Learning, Artificial Neural Networks

An ANN or Genetic-Algorithm-ANN was trained to correlate cell count in a *C. reinhardtii* cultivation measured with a hemocytometer with the fluorescence emission intensity of suspension samples at 101 wavelengths acquired in the range 660–760 nm with 1 nm steps, excited with a 470 nm LED [39]. In total, 1568 fluorescence spectra at changing cell concentrations were acquired. Intensity of the fluorescence peak was used as input data. With increasing cell concentration, the peak moved to a slightly longer wavelength. The cell concentration range was 2×10^5–6.4×10^6 cells·mL^{-1}. Comparison of the measured and predicted cell count values in three validation sets showed an acceptable result only in one set, with errors in the range −8 to 34% of the true (hemocytometer measured) value, where the other two sets had prediction errors in the range 5 to 110% and −95 to 25% of the true value.

An ANN trained with light absorption data could differentiate between monoalgal and mixed algal cultures and identify contamination of a single strain culture by another species [65] (for more detail, see Section 2.4).

Lopez-Exposito et al. [23] measured reflectance and processed data by a perceptron (ANN) estimating the biomass concentration with a correlation coefficient of $R^2 = 0.92$. In [24], reflectance data processed by two chemometric models estimated the biomass concentration with accuracy of $R^2 = 0.81$–0.87 (for more detail, see Section 2.3.4).

4.4. Chemometric Models

Changes of protein, lipid and carbohydrate content were monitored in freeze-dried biomass samples of seven green algae strains grown under nitrogen starvation using ATR-FTIR spectroscopy [87]. Spectral intensities in the preselected diagnostic bands, determined as the area under the peak, were processed by three statistical methods (ULRA, OPLS, MCR-

ALS) to obtain correlations between component fraction in biomass, measured by standard analytic methods, and the corresponding spectral intensity, with the resulting R^2 between the estimated and measured values 0.65/0.77/0.63 (carbohydrates, ULRA/OPLS/MCR-ALS), 0.82/0.90/0.77 (lipids, ULRA/OPLS/MCR-ALS), 0.88/0.92/0.85 (proteins, ULRA/OP LS/MCR-ALS) and 0.84 (ratio carbohydrates/proteins, only ULRA).

In a series of publications, Sa and various co-authors [40,41,48–50] and other authors [32,63] developed data-driven chemometric models based on 2D fluorometry (EEMs) as inputs for estimation of several biological variables in microalgal cultivations, including cell count, cell viability and concentrations of nitrate, chlorophylls a and b, carotenoids, fatty acids as total, saturated and unsaturated, and eicosapentaenoic acid fraction in the TAG content. Such models could potentially be used for real time on-line estimation of various process variables by acquiring the real time data used as input for models automatically on-line through suitably designed and placed fiber probes measuring fluorescence just as they are used in the series for measuring samples drawn from the PBR.

5. Perspectives and Outlook for On-Line Sensing in Microalgal Cultivations

When comparing the current state of instrumentation suitable for on-line measurements to the situation several years ago [10], the availability of miniaturized and rugged spectrometers in all wavelength ranges, from UV over VIS to NIR, equipped also for Raman and fluorescence measurements, has substantially increased, together with miniaturized fiber optics. This opens the path for relatively easy construction of spectroscopic optical sensors enabling fast on-line multispectral measurements providing signals for processing in real-time applications and should support the use of optical sensing when possible: optical sensors are non-invasive, fast, robust with no moving parts, and rather low-priced compared with non-optical methods. The miniaturized fiber optics allows flexible placement of sensing spots. On-line monitoring of biological variables as lipids, pigments and other biomass components, contamination and pathogens and physiological state of microorganisms in microalgal cultivations could thus rely on a plethora of signals from physical sensors as inputs for combinations of chemometric models, mathematical process models and pattern and image recognition tools integrated into software sensors providing data about the current concentrations of biomass, biomass components, culture fitness and contamination and pathogen dangers. Recent developments in microalgal technology, leading to the complete use of all microalgal biomass components in a biorefinery, calls also for sensors, software or physical, suitable for monitoring various microalgal biomass components. Recently, the focus in microalgal cultivations has shifted from the production of single components to maximizing the biomass production and productivity with subsequent complete processing of the produced biomass in a biorefinery so that real-time monitoring of culture fitness and the culture's physiological state and quantum yield [42,44,45] are very important. Most microalgal biomass is produced in open photobioreactors where contamination by various pathogens, e.g., grazers, poses a grave danger. Here, an early detection of contaminating organisms is very important and the in-situ microscopy with automatic image evaluation, reflectance, multispectral absorbance, color measurement or FTIR spectroscopy could be employed [22,33,67,90]. Microfluidics is another technology finding its place in microalgal research and industrial applications in monitoring and optimizing both upstream and downstream processes, performing functions in cell sorting, screening and characterization, cultivation and lipid and pigment identification using measurement methods as intrinsic fluorescence and fluorescence after staining, Raman spectroscopy, dielectrophoresis or magnetophoresis [91]. It has already been implemented in on-line monitoring of biomass by fluorescence [37,58] or for sorting cells based on their lipid content [57].

6. Conclusions

The most widely used proxy for biomass concentration, measurement of optical density or turbidity by light absorption or scattering, works reliably with standardized

industrial sensors. Some of the current off-line monitoring methods for process products and biological characteristics could be adapted for on-line and at-line application, preferably those based on optical measurement principles as fluorescence, spectroscopy in visible and infrared ranges and multispectral reflectance. Using fiber optic cables enables the non-invasive application of various spectroscopic sensors in situ when necessary. Software sensors using signals of physical sensors as inputs and processing and combining the signals of physical sensors employing chemometric models, mathematical process models and powerful pattern recognition tools as artificial neural networks can provide real-time estimates of biological process variables with accuracy comparable to a direct off-line measurement. Software sensors can also be easily adjusted to include new signal sources, i.e., new hardware sensors available and applied to the process, in the estimation of biological process variables. This would mean lowering the price for development of new sensors—in this case software sensors—for biological process variables in industrial applications, where costs are always a matter of concern.

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Abbreviations

ANN	artificial neural networks
ATR	attenuated total reflection (infrared spectroscopy)
BC	biomass concentration
BODIPY	boron-dipyrromethene, a fluorescent dye
CARS-MLR	competitive adaptive reweighted sampling MLR
CC	cell count
CCD	charge coupled device
CLD	chord length description
DWC	dry weight concentration (of biomass)
EC	extinction coefficient
EEM	excitation-emission matrix
EKF	extended Kalman filter
EX	extinction coefficient
FAME	fatty acid methylester
FBRM	focused beam reflectance measurement probe
FID	flame ionization detector
FTIR	Fourier transform infrared-spectroscopy
GA-ANN	genetic algorithm - artificial neural networks
GC	gas chromatography
HR	hyperspectral reflectance
IR	infrared
ISM	In-Situ Microscope
LED	light emitting diode
LW-PLS	locally weighted PLS
MCR-ALS	multivariate curve resolution - alternating least squares
MLR	multiple linear regression
MPC	model predictive control

MS	mass spectrometry
NIR	near infrared radiation
NIRS	NIR spectroscopy
NMR	nuclear magnetic resonance
N-PLS	multilinear PLS
NPQ	non-photochemical quenching
NTU	nephelometric turbidity unit
OD	optical density
OPLS	orthogonal partial least squares
PAH	polycyclic aromatic hydrocarbons
PAM	pulse amplitude modulation, a fluorescence technique
PAR	photosynthetically active radiation
PBR	photobioreactor
PCA	principal component analysis
PCR	principal components regression
PDMS	polydimethylsiloxane
PE	photosynthetic efficiency
PF	particle filter
PLS	partial least squares (regression)
PQY	photosynthetic quantum yield
r	Pearson correlation coefficient
R^2	coefficient of determination in a regression
RF	random forest (regression)
RGB	red-green-blue (color description model)
SPA-PLS	successive projections algorithm PLS
SVR	support vector regression
T	transmittance
TAG	triacylglycerides
TLC	thin layer cultivation
UKF	unscented Kalman filter
ULRA	univariate linear regression analysis
UV	ultraviolet
UVE-PLS	uninformative variable elimination PLS
VIS	visual (range of radiation)
WATERGATE	WATER suppression by GrAdient Tailored Excitation, an NMR technique
$\Delta F'/F_m'$	effective quantum yield (in PAM fluorometry)
ε	permittivity
ρ	Pearson correlation coefficient

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