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► To cite this version:

Dylan Bouillaud, Benoît Charrier, Delphine Drouin, Corentin Jacquemmoz, Jonathan Farjon, et al.. Using benchtop NMR spectroscopy as an online non-invasive in vivo lipid sensor for microalgae cultivated in photobioreactors. *Process Biochemistry*, 2020, 93, pp.63 - 68. <10.1016/j.procbio.2020.03.016>. <hal-02999895>

HAL Id: hal-02999895

<https://hal.science/hal-02999895v1>

Submitted on 11 Nov 2020

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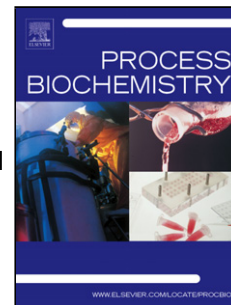


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PII: S1359-5113(20)30087-8

DOI: <https://doi.org/10.1016/j.procbio.2020.03.016>

Reference: PRBI 11968

To appear in: *Process Biochemistry*

Received Date: 24 January 2020

Revised Date: 9 March 2020

Accepted Date: 21 March 2020

Please cite this article as: Bouillaud D, Drouin D, Charrier B, Jacquemmoz C, Farjon J, Giraudeau P, Gonçalves O, Using benchtop NMR spectroscopy as an online non-invasive *in vivo* lipid sensor for microalgae cultivated in photobioreactors, *Process Biochemistry* (2020), doi: <https://doi.org/10.1016/j.procbio.2020.03.016>

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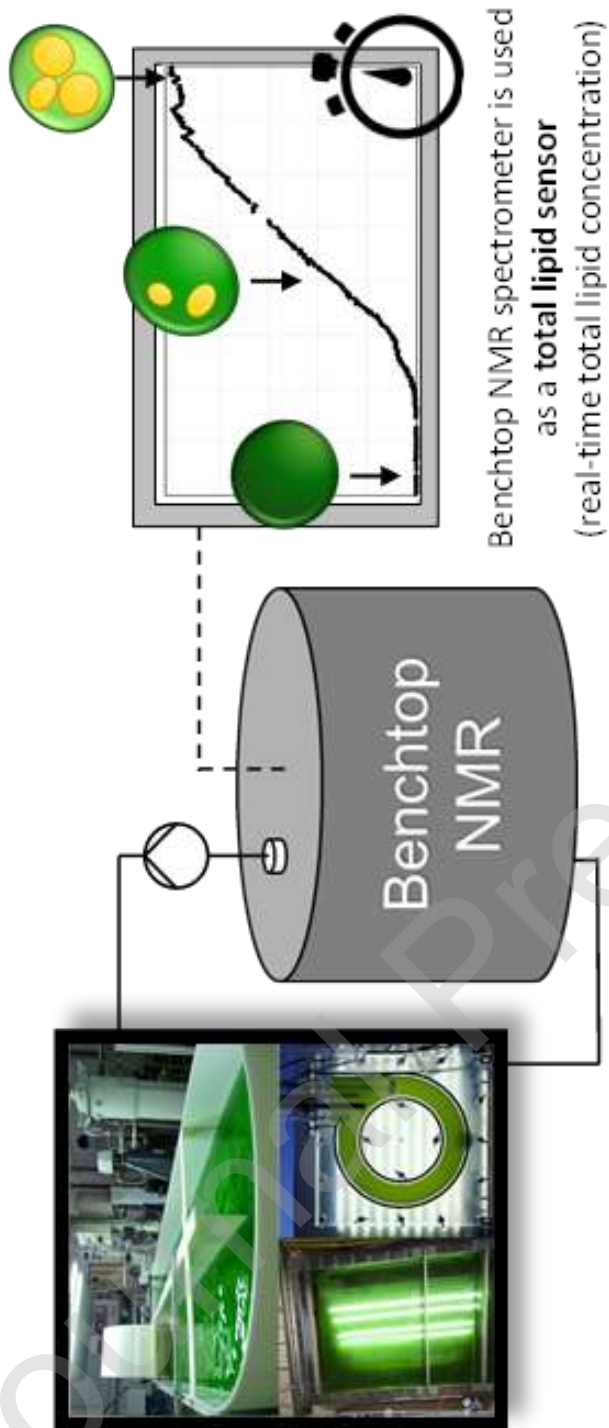
**Using benchtop NMR spectroscopy as an online non-invasive *in vivo*
lipid sensor for microalgae cultivated in photobioreactors**

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Graphical abstract



Highlights

- Compact NMR spectroscopy as an online non-invasive microalgae *in vivo* lipid sensor
- The NMR signal is quantitative when calibrated with total lipid reference method
- The NMR performance is compatible with the bioprocess operating conditions
- Lipid productivities are measured in real-time for real-time optimization purposes

Highlights

- Benchtop NMR spectroscopy is a suitable microalgae lipid sensor.
- Microalgae intracellular lipid monitoring is feasible without any human intervention.
- Sensitivity limits are compatible with real microalgae bioprocesses.

Abstract

The production of lipids by microalgae is widely studied, especially to find the best bioprocess operating conditions and optimize the productivity of the targeted product. In this context, being able to monitor online the evolution of the lipid concentration is a great advantage regarding the control and/or the optimization of the production. Yet, most non-invasive analyses hit a brick wall on the interference of the

lipid signal with the ubiquitous water of the culture medium. This article shows how a compact NMR spectrometer connected to a photobioreactor can circumvent this drawback and measure, in real-time and in a non-invasive manner, the total lipid concentration, and that directly on the entire cells grown in their culture medium. The water signal could be enough-selectively removed using the W5 version of the WATERGATE pulse sequence. The NMR signal nicely correlates ($R^2 > 0.99$) with the offline FAME (Fatty Acid Methyl Ester) total lipid analysis as performed by GC-FID (Gas Chromatography coupled to Flame Ionization Detector) within limits of detection and quantification of respectively 9 and 30 mg.L⁻¹. The lipid specific signal appears also quite robust regarding the dissolved dioxygen, making the benchtop NMR spectroscopy an appropriate universal device for the online monitoring of lipids produced in bioprocesses.

Keywords

microalgae, benchtop NMR spectroscopy, photobioreactor, lipid online monitoring

1. Introduction

The production of lipids by microalgae is one of the most studied topics by a substantial research and industrial community since it is involved in several global issues: biofuel or edible oils can be produced in order to substitute, at least partially, the current ways of production for sustainable development purposes [1–3]. Some microalgae species are known for their ability to produce lipids: a metabolic shift is provoked when they are cultivated in a nitrogen-free medium, leading to the biosynthesis of reserve lipid (mainly triglycerides). Finding the best lipid production conditions is a long-term endeavor from the identification of the best strain to the industrial production by the way of the scale-up steps.

One of the main limitations of these studies is access to the *in vivo* lipid information. It is often reported that an online intracellular lipid sensor for microalgae cultures is needed [4,5]. It would help in understanding and measuring real-time effects on lipid generation (e.g. day/night cycles) and allow better control for lipid production purposes. A lot of methods have been developed and can bring lipid information in various ways and in various timescales. The first step often consists in a lipid extraction following the recommendations of Bligh and Dyer [6] or Folch et al. [7]. Once extracted, the total lipid content can be estimated gravimetrically or using different analytical techniques. The most widely used is undoubtedly the FAME analysis through (Fatty Acid Methyl Ester) GC (Gas Chromatography) which allows –after a transesterification step– the whole qualitative and quantitative assay of lipid chains [8–10]. Lipids can also be analyzed using other chromatography techniques such as TLC or HPTLC [11,12] (Thin Layer Chromatography) or HPLC [13] (High Performance Liquid

Chromatography). These chromatographic techniques can be hyphenated with MS (Mass Spectrometry) detection [14–16]. This is a non-exhaustive list for the analysis of the extracted lipids, but these offline techniques all require considerable amount of time. In order to partially overcome this problem, techniques working on raw material can be employed to propose a quick estimation of the lipid content: Nile red staining with fluorimetry [17,18], infrared spectroscopy [10,19], Raman spectroscopy [20], MS (through Matrix Assisted Laser Desorption Ionization interface) [21] or TD-NMR [22,23] (Time-Domain Nuclear Magnetic Resonance). However, the use of these techniques as online flow sensors has been limited so far to fermentation bioprocesses with very high biomass concentrations. Moreover, they often require considerable bioprocess adjustments coupled with hard processing approaches [24] because of different analytical barriers, the main one being the analysis of dilute analytes in aqueous media.

NMR spectroscopy is known as a highly reproducible technique and can be adapted to various samples thanks to the wide range of pulse sequences developed by the NMR community [25,26]. Pulse sequences are sets of radio frequency pulses designed to excite, suppress, or manipulate specific nuclei. A class of pulse sequences are designed to observe analytes whose signals cannot be detected because of the very strong solvent signal and are called solvent suppression pulse sequences. Initially performed on massive and unmovable devices, NMR spectroscopy experiments have become available at a reduced scale (called benchtop NMR [27,28]) thanks to recent hardware developments. This opens up the scope of possibilities in process monitoring since the analysis is flow-compatible and the apparatus can henceforth be placed as close as possible to the process. The implementation of a gradient coil in the hardware

[29] makes it possible to implement, gradient-based pulse sequences such as those capable of performing solvent suppression. Recently, we reported a proof-of-concept illustrating that benchtop NMR spectroscopy could be used as a flow online lipid-selective sensor [30]. In this study, three independent cultures of *Nannochloropsis gaditana* were analyzed in flow conditions. The three cultures presented three different lipid concentrations which were clearly distinguishable with benchtop NMR thanks to an optimized water suppression pulse sequence called W5 [31]. The good repeatability and the good agreement with the FAME analysis were promising data in order to prove the ability of benchtop NMR to monitor a whole microalgae culture. However, these cultures were rather static from a biological point of view since they were analyzed in a short timeframe. The online non-invasive coupling between NMR and a microalgae culture evolving with time in real conditions has never been performed to date.

In the present work, the coupling between a benchtop NMR spectrometer and a photobioreactor (PBR) running a microalgae culture driven for lipids production was successfully performed for the first time. The industrial strain, *Parachlorella kessleri* was grown under nitrogen starvation in a torus PBR and its total lipid accumulation was monitored online for 400 hours. The real-time lipid monitoring was performed in a non-invasive way, directly on the culture broth. The coupling between the PBR and the spectrometer was performed using a circulation loop (online analysis), presenting a negligible active volume regarding the total volume of the culture, so the setup was non-invasive regarding the illumination strategy, i.e. provoking no culture shading zone. The results and the perspectives emerging from this study are described and discussed hereafter.

2. Material and methods

2.1 Microalgae and photobioreactor

The *Parachlorella kessleri* strain (UTEX2229) was obtained from the algae culture collection at the University of Texas. Microalgae were previously grown in a modified Bold Basal Medium (BBM). The medium composition is described in the supplementary Material (Table S1). 150 mL of microalgae culture were used for the PBR inoculation.

The cultivation was performed in a 1.9 L torus PBR with a thickness of 5.3 cm (outer diameter: 26 cm and inner diameter: 17.5 cm). The shape of this PBR was designed to provide a homogeneous cultivation, and therefore homogeneous cell samples [32]. The temperature was regulated at 25°C and the pH at 8 by adjusting the CO₂ inlet flow (proportional–integral–derivative controller), also providing the CO₂ for the growth of the algae. The air was also continuously injected in order to desorb the dissolved O₂ produced during the photosynthesis and for stirring purpose as well. The PBR was equipped with O₂, pH and temperature probes. The illumination was carried out by a light LED panel which was previously calibrated using a quantum light sensor. The incident photon flux density was fixed at 200 $\mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

After a sterilization step, 150 mL of pre-cultured *Parachlorella kessleri* were inoculated in the PBR, reaching an initial biomass concentration of 0.034 g.L⁻¹. The culture medium in the PBR was identical to that of the inoculum (Table S1) except for the nitrate concentration which was decreased at 4 mmol.L⁻¹ in order to perform a progressive starvation: first a growth stage followed by a starvation stage, provoking the

lipid accumulation once all the nitrogen was consumed. The data were collected for 400 hours.

2.2 *Benchtop NMR*

The benchtop NMR device was a Spinsolve $^1\text{H}/^{19}\text{F}/^{13}\text{C}$ from Magritek delivering a 1.02 T magnetic field corresponding to a proton frequency of 43.5 MHz. The device was equipped with a gradient coil along the B_0 direction, allowing to perform the solvent suppression pulse sequence called W5, which was optimized in our previous work [30]. This pulse sequence is an improvement of the original WATERGATE pulse sequence [31,33]. The pulse sequence parameters are detailed in the supplementary material (Figure S2). The duration of the hard 90° pulse was tuned at $11.3 \mu\text{s}$ with a power attenuation of 0 dB. Through the SpinsolveExpert software (version 1.25), a total of 310 spectra were acquired. Each spectrum was acquired for one hour and consisted of an accumulation of 3600 scans with a 1 s repetition time. Each scan was recorded with 4096 points and a dwell time of $50 \mu\text{s}$ resulting in an acquisition time of 204.8 ms. The one hour duration was chosen as a good compromise between the sensitivity of acquisition and the biological timescale. 90 missing data points were due to the daily shimming procedure to preserve the magnetic field homogeneity as well as two unwanted interruptions of the acquisition around 65 and 260 h of culture.

The spectra were processed with the MestReNova software (version 12.0): a zero-filling factor of 32 was applied (128k final points) a manual phasing and a manual baseline correction (Whittaker) were performed on each spectrum. No apodization function was applied. The lipid signal region between 0.5 and 1.3 ppm was integrated.

The coupling between the PBR and the benchtop spectrometer was performed through a small loop of 11 mL so the volume of the loop was negligible compared to the volume of the whole culture. A peristaltic pump was used to make the cultivation going through the sensitive volume of the NMR. The flow rate of this loop was set to 2.0 mL.min⁻¹ in order to ensure the homogeneity inside the loop while remaining compatible with the NMR acquisition on a flowing sample. A picture of the setup is shown in Figure 1.

2.3 *Offline analyses performed on the culture*

In addition to the online NMR measurement, mandatory parameters were measured at specific timepoints, first for monitoring classical parameters of the culture and second to compare the NMR results with a reference technique used for the quantification of the total lipids. Indeed, the NMR parameters (fast pulsing and use of the W5 pulse sequence) require an external calibration. A volume of the culture was removed daily and immediately analyzed or stored at -20°C for further gas and ionic chromatography analyses. It was modulated to fit with the minimum quantity of matter requirement of each analytical methods. It was also modulated to preserve the minimum required volume for the PBR: 1.5 L at the end of cultivation.

The dry weight contents were measured by filtering a volume of culture through a dried glass-fiber filter (Whatman GF/F). The filters were dried 24 hours at 105°C before weighing. The mass difference between before and after the filtration was used to determine the biomass dry weight. No replicate could be made because of the limited quantity of available biomass.

The turbidity of the culture was also monitored in order to obtain a quick assessment of the biomass concentration using limited amount of material. The optical density of the culture at 750 nm was used, measured using a spectrophotometer CARY 5E UV-VIS-NIR (Agilent).

The cell numbering was performed using a Malassez hemocytometer under optical microscope after an appropriate dilution.

The pigment concentration was determined after an extraction in analytical grade methanol in the dark for 1 h at 45 °C. The cell residues were removed after a centrifugation step and the supernatant was analyzed by a spectrophotometer CARY 5E UV-VIS-NIR (Agilent). The absorbances at 480, 652, 665 and 750 nm were then measured. The absorbance at 750 nm was used to confirm the elimination of the solid particles. Then, the Ritchie equations were used to determine the concentrations of the chlorophyll a C_{chlA} , chlorophyll b C_{chlB} (Eqs. 1 and 2) [34] and Strickland and Parsons's equation for the estimation of the concentration of the carotenoid pigments C_{carot} (Eq. 3) [35].

$$(1) \quad C_{chlA} = -8.10 \times A_{652} + 16.52 \times A_{665}$$

$$(2) \quad C_{chlB} = 27.44 \times A_{652} - 12.17 \times A_{665}$$

$$(3) \quad C_{carot} = 4.0 \times A_{480}$$

The FAME analysis was performed after freeze-drying, extraction and transesterification steps, through a dedicated protocol using the gas chromatography (Agilent 7820) equipped with a TR-FAME column (30m x 0.25mm x 25 μ m Thermo-Fisher) and a flame ionization detector. The full protocol was detailed in the

supplementary material of our previous work [30]. Each measurement was made in triplicate.

The nitrate concentration was determined in the culture medium through an ionic chromatography system including an anionic chromatograph (Dionex-ICS 900-IonPac) equipped with a AG9-HC guard column, a AS9HC separation column, and an external AMMS (Anion-ICE MicroMembrane Suppressor 300, Dionex) supplied with sulfuric acid (H_2SO_4 , 25 mmol.L^{-1} , 1.8 mL.min^{-1}). The eluent was a solution of 7.7 mmol.L^{-1} Na_2CO_3 and 1.3 mmol.L^{-1} NaHCO_3 at a flow of 1 ml.min^{-1} . The detection was performed by conductivity, and the data acquisition and processing were performed using the Chromeleon software, version 7.0. The samples were filtered to 0.45 μm before measurement by ionic chromatography.

3. Results and discussion

3.1 ^1H NMR spectra

An overview of the recorded spectra is presented in Figure 2. In our previous work [30], we showed that the $-\text{CH}_2-$ located in the middle of the saturated fatty chains as well as the terminal $-\text{CH}_3$ could be observed on the ^1H NMR spectra recorded on entire cells. These protons are in majority among lipid chain protons and can be therefore related to the total lipid amount in the microalgae cells [30]. This statement will be further verified a second time in section 3.3 through the comparison with the FAME analysis results. The peak situated at 1.2 ppm with its shoulder at 0.9 ppm presents a time-dependent evolution (increase) for the whole duration of the experiment (Figure 2). The residual water peak after the suppression can be observed at 4.7 ppm.

3.2 Culture monitoring

The collected data in Figure 3 indicates that the starvation and the lipid accumulation were effective and confirms that the analyses are consistent with similar cultivation experiments described in the literature [10,36]. At the beginning of the culture, a growth phase can be observed, during which the microalgae plentifully multiply themselves (pigments, turbidity, dry matter and cell concentration important increases) while there is still enough nitrogen left (nitrate) i.e. until c.a. 100 hours of cultivation. A progressive starvation is carried on in this study, so a transitional stage can be observed between 100 and 200 hours. The modulation of the pigment concentration can be explained by the modulation of the photosynthetic efficiency, provoked by the beginning of the starvation. The cell multiplication is progressively stopped and the dry matter concentration tends towards a threshold (c.a. 200 h). Concomitantly, lipid accumulation can be observed with both chromatographic and spectrometric techniques. For c.a. 200h of cultivation a strong decrease of the pigment concentration can be observed, as well as in cell concentration because cells are no longer renewed as typically observed in nutrient deprivation experiments [10]. Indeed, the metabolism of the cultivated microalgae is no more solicited towards the biomass production but towards the synthesis of energetic reserve molecules (i.e. the lipids).

From a statistical point-of-view, it was not possible to perform replicates for all analyses because of the limited retrievable volume of culture (400mL for the whole experiment). It is not critical for the analyses which were used as culture indicators because their relative evolution is more relevant than the absolute values. Regarding the FAME analysis, it is mandatory to check that the short-term good linearity between the

NMR signal with the total lipid concentration presented in our previous work [30] is still valid in this long-term monitoring so FAME analyses were performed in triplicate.

3.3 Comparison of the total lipid determination using NMR and GC-FID methods

In order to evaluate the ability of the online NMR signal to bring information about the total lipids, the NMR data were compared with the data from the FAME analysis performed off-line by GC-FID. Figure 3 illustrates that both techniques show the same relative evolution all along the duration of the experiment. A slight deviation is noticeable at the beginning of the experiment, due to the lower limit of detection of the GC-FID method compared to NMR. Nevertheless the results clearly indicate that the online benchtop NMR is a reliable tool to monitor in real time the evolution of the lipids on entire microalgae cells, in a non-invasive and online fashion. As demonstrated previously [30], the high reproducibility of the NMR approach ensures that the integrated signals provide a reliable estimation of the total amount of aliphatic proton groups in the whole analyzed culture samples.

In order to verify that the evolution of the above mentioned alkyl protons is still representative of the evolution of total lipids, as it was the case in our previous work [30], NMR data are compared with GC-FID data in Figure 4. The calculated linear correlation (least square regression, $R^2 > 0.99$) clearly indicates that the NMR signal is a consistent indicator of the total lipid concentration. It should be noticed that the FAME analysis is applied on a certain mass of dry biomass whereas the NMR detects the concentration in a volume of culture. Therefore, the lipid content determined by GC-FID were converted in lipid concentration in the culture by multiplying by the dry weight content, in order to be comparable.

The online NMR results are in very good agreement with those from the offline reference method. Using this correlation and based on the NMR SNR (Signal-to-Noise Ratio), the LOD and LOQ were respectively estimated at 9.0 and 30 mg.L⁻¹ of total lipids corresponding to a SNR of 3 and 10, which is compatible with the classical operating parameters of most bioprocesses. A very important statement about all these specifications need to be kept in mind: it is possible to adjust those limits by tuning the acquisition parameters: a better sensitivity would be obtained by accumulating more NMR scans for example, albeit at the cost of a lower number of points in the time dimension.

3.4 Interest for bioprocess applications

Using the previously described excellent linear correlation, it is correct and relevant to convert the lipid signal directly into lipid concentration so that the benchtop NMR spectrometer can be considered as a lipid sensor. Dividing the concentration by the time of cultivation, the lipid volumetric productivity can be calculated. This one is then converted into areal productivity thanks to the illuminated specific area (19 m²). In Figure 5 are compiled the real-time lipid concentration and their areal productivity. The lipid productivity increases with time until 1.7×10⁻³ kg.m⁻².day⁻¹ around after 330 h of culture, the overall values are of the same order of magnitude than the calculated maximum productivities reported by Taleb et al. (2018) on the same strain (between 2.7 and 4.4×10⁻³ kg.m⁻².day⁻¹) but with the reference measurement methods. The slight difference observed here can be explained by the better illuminated specific area (33 m²) of their photobioreactor. In comparison to this paper where only a few points were measured, accessing the real-time productivity could provide very interesting perspectives as a way to better characterize and understand different effects that the

authors investigated, such as the day/night cycles, the light transfer or the effect of the concentration of the inoculation.

An expected limitation that can negatively impact the online NMR detection is the unavoidable production of dioxygen occurring during the cultivation of the microalgae. The dioxygen is indeed a paramagnetic agent which could impact the transverse relaxation times of lipids, causing a modification of the detected NMR signal. Here, the dioxygen concentration was monitored all along the duration of the experiment (expressed in % compared to air saturation), since it is also a good indicator of the cell multiplication. Moreover, the desorption of the dissolved dioxygen was forced using injection of air in the PBR but in this experiment it was not controlled so that the oxygen concentration was not constant. Therefore, it was expected that variable dissolved dioxygen concentration could have disrupted the NMR lipid monitoring. In order to evaluate this perturbation, the dioxygen concentration and the NMR lipid signal kinetic evolution between 350 and 400 hours of cultivation are compared (Figure 6). An air interruption occurred around 359h due to a technical failure of the air system, which made the dissolved dioxygen concentration rapidly increasing from 170% to 300% in less than one day. The effect on the NMR lipid signal is clearly observable in an opposite way than the dissolved dioxygen increase which cannot be attributed to a biological evolution. It is yet interesting that the signal decreases not more than 4% between 170 and 300% of dissolved dioxygen concentration, which highlights that the lipid signal is robust, even towards dioxygen concentration variations and that even if the dioxygen is not controlled, which is the case for most of the high-scale bioprocesses.

3.5 Perspectives

This device can be considered as a lipid probe, which is able to monitor a parameter but must be calibrated in order to provide absolute quantification. In the case of further coupling with different bioprocesses (including the extension to other living system cultivations), the matrix effect is something that need to be carefully monitored because this implies either to calibrate the NMR signal with a reference technique for each coupling or to ensure that the analysis is reproducible enough to always provide the same signal for an identical lipid concentration whatever the conditions.

No extraction and derivatization steps -which cause cumulative errors and are time consuming- are needed. Only three quick processing steps are mandatory: phase correction, baseline correction and integration which could be automatized in the future to reduce the human time. In practical terms, the apparatus can be placed as close as possible to the bioprocess system (here a torus PBR). Only a small loop is needed to connect it to the bioprocess.

From an NMR methodology point of view, several investigations could be interesting to perform. NMR parameters could be slightly optimized such as the use of Ernst angles (allowing faster pulse rate with small angles) in adapting the pulse sequence. Moreover, a deeper investigation of the effect of the dioxygen concentration on the lipid signal could be performed by measuring relaxation times over the course of experiment. Such an interleaved relaxometry/spectroscopy would help to better understand and possibly correct their effects.

The advantages of the technique are substantial compared to usual methods. First NMR is used as an online sensor, providing the result in real-time (one point every hour can be considered as real-time regarding the whole culture duration). In a lipid

production context for example, it can provide the real-time lipid productivity and can indicate the best moment to harvest the microalgae or calculate and compare the bioprocess productivities. The present results clearly open the route for real-time optimization (RTO) approaches using benchtop NMR spectrometers as robust sensors. Another advantage of this approach is its low cost of analysis; arising from the relatively low cost of the equipment itself (50-100 k€ range) and from the absence of maintenance costs. Based on a 5-year depreciation period with an occupancy rate of 75%, this leads to a cost of analysis of 2.50 € per hour.

These results pave the way towards the implementation of such monitoring on an industrial or semi-industrial scale, even if some precaution still need to be taken into account. For example, in the case of heterogeneous cultures where microalgae can settle, it may pose a problem of representativeness of the acquired data. The device is also quite sensitive to room temperature variations, so it is mandatory to operate it in a dedicated temperate room (or mobile shelter) with limited temperature variations. The NMR detection could also be coupled with other kind of sensors (cytometry, Raman or NIR sensors, ...) to combine multiple sources of real-time information. Finally, benchtop NMR spectroscopy is a recent technology and the ongoing developments (such as the increase in magnetic field strength and homogeneity) will undoubtedly improve the performance of the method in terms of limit of detection and resolution.

4. Conclusion

The present work demonstrates that compact NMR spectroscopy is able to provide a real-time signal which is representative of the total lipid concentration. The relative evolution of this signal allows the *in vivo* non-invasive monitoring of the total

lipids on microalgae grown under bioprocesses real conditions. Furthermore, the comparison of the NMR data with a quantitative total lipid analysis makes the NMR analysis quantitative, so lipid concentration and productivity values are calculated in real-time. In this study conditions, the limit of detection was measured at 9 mg.L⁻¹ and this limit can be tuned in the case of different bioprocesses.

Declaration of competing interest

The authors declare that they have no conflict of interest

Acknowledgments

The authors acknowledge support from the Region Pays de la Loire (“Pari Scientifique Régional AMER-METAL”), the French National Center for Scientific Research (“Osez l’Interdisciplinarité !” RMN-(ME)2- TAL) and from the CORSAIRE metabolomics facility. Lenaïc Lartigue and Elena Ishow are warmly thanked for their help to use the spectrophotometer and microscope. J.F. thanks his partner Sandrine Bouchet for an unfailing assistance.

Author contributions

DB performed most experiments: microalgae cultivation, photobioreactor launch and monitoring, extraction, analyses except gas and ionic chromatography, NMR data analysis and wrote the article. DD performed gas and ionic chromatography analyses. BC was in charge of technical aspect. CJ automatized NMR process steps and result compilation. OG, JF and PG critically revised the article. All authors read and approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version of the paper.

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Legends

Figure 1. Picture of the coupling between a PBR (*Parachlorella kessleri* culture) on the left and a benchtop spectrometer (grey) on the right using a peristaltic pump (highlighted in blue). The loop connects the PBR with capillaries (accentuated in full green line) to a smooth quartz tube (accentuated in dashed green line). The sensitive volume of NMR is represented in red.

Figure 2. 3D stacked plot of half the 310 ^1H NMR spectra for the sake of readability, after phase and baseline corrections. The residual water peak at 4.7 ppm and the significant growth of the main lipid peak at 1.2 ppm over the course of the cultivation are highlighted.

Figure 3. Time-dependent evolution of the biomass monitoring parameters during the progressive nitrogen deprivation protocol. All the results were normalized to their highest value. The numerical non-normalized results are presented in the **supplementary material (Table S.3)**. A slight deviation is observed between the two lipid detection techniques because both techniques do not present the same limits of detection. Time 0, corresponds to the inoculation of the culture. The normalized units were initially expressed in g.L^{-1} for the dry weight concentration, in number of cells per mL for the cell concentration, in absorbance units for the turbidity, in $\mu\text{g.mL}^{-1}$ for the pigment concentration, in mg.L^{-1} for the nitrate concentration, in mg.L^{-1} of total lipids for the lipid FAME analysis by GC-FID (error bars correspond to the standard error on 3 replicates) and in arbitrary units for the NMR signal. Discontinuities in the NMR signal (observable at the end of the experiment) are owed to the shimming procedure after the slow deviation of the magnetic field homogeneity.

Figure 4. Linear relative correlation of the results from the two lipid analyses i.e. GC-FID and benchtop NMR spectroscopy. The selected benchtop NMR data points were kinetically the closest from those of the extractions performed for the GC-FID analysis. The linear regression was performed on 10 points (red crosses) and presented a determination coefficient of 0.996, highlighting the very good estimation of the total lipid concentration by the benchtop NMR approach. As both techniques do not provide the same limits of detection, the linear regression does not go through the origin and the three first points (in blue, for which the NMR signal was null) were not taken into account for the regression. Vertical error bars represent the standard error on three consecutive NMR points (assuming that the biological evolution is negligible). Horizontal error bars represent the standard error on three GC-FID replicates.

Figure 5. Lipid concentration over the time of cultivation: the lipid NMR signal was converted in lipid concentration (in red) using the correlation with FAME analysis presented in Figure 4. This allows the calculation of the real-time areal lipid productivity of the cultivation (in blue). Before 90h, the NMR lipid signal is null.

Figure 6. Effect of paramagnetic dioxygen on NMR lipid signal, an air interruption occurred between 359h and 392h (blue stripped lines). The induced increase of dissolved dioxygen made the NMR signal weaker. The discontinuity at 383h is due to a culture extraction (dotted green line).

Graphical abstract

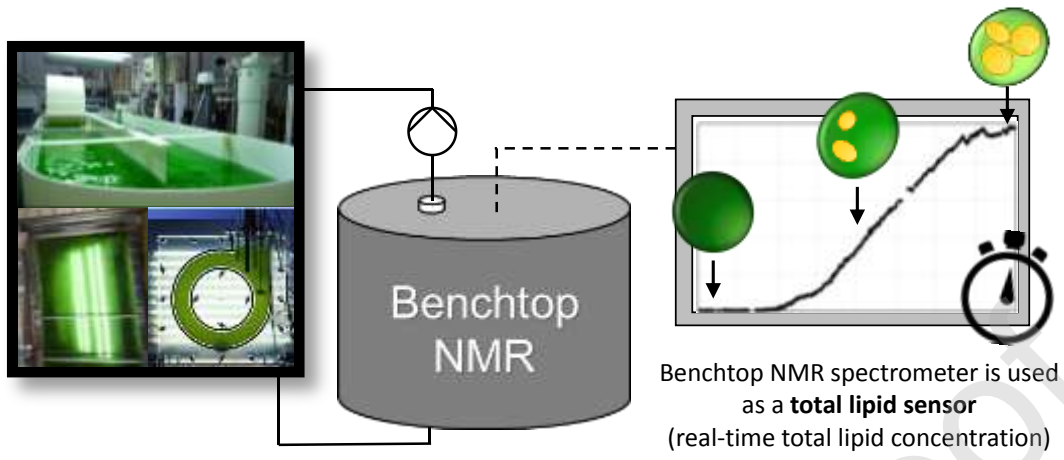
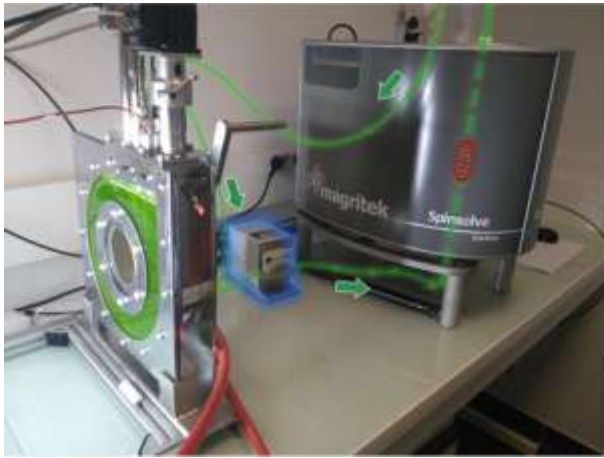


Figure 1



Journal Pre-proof

Figure 2

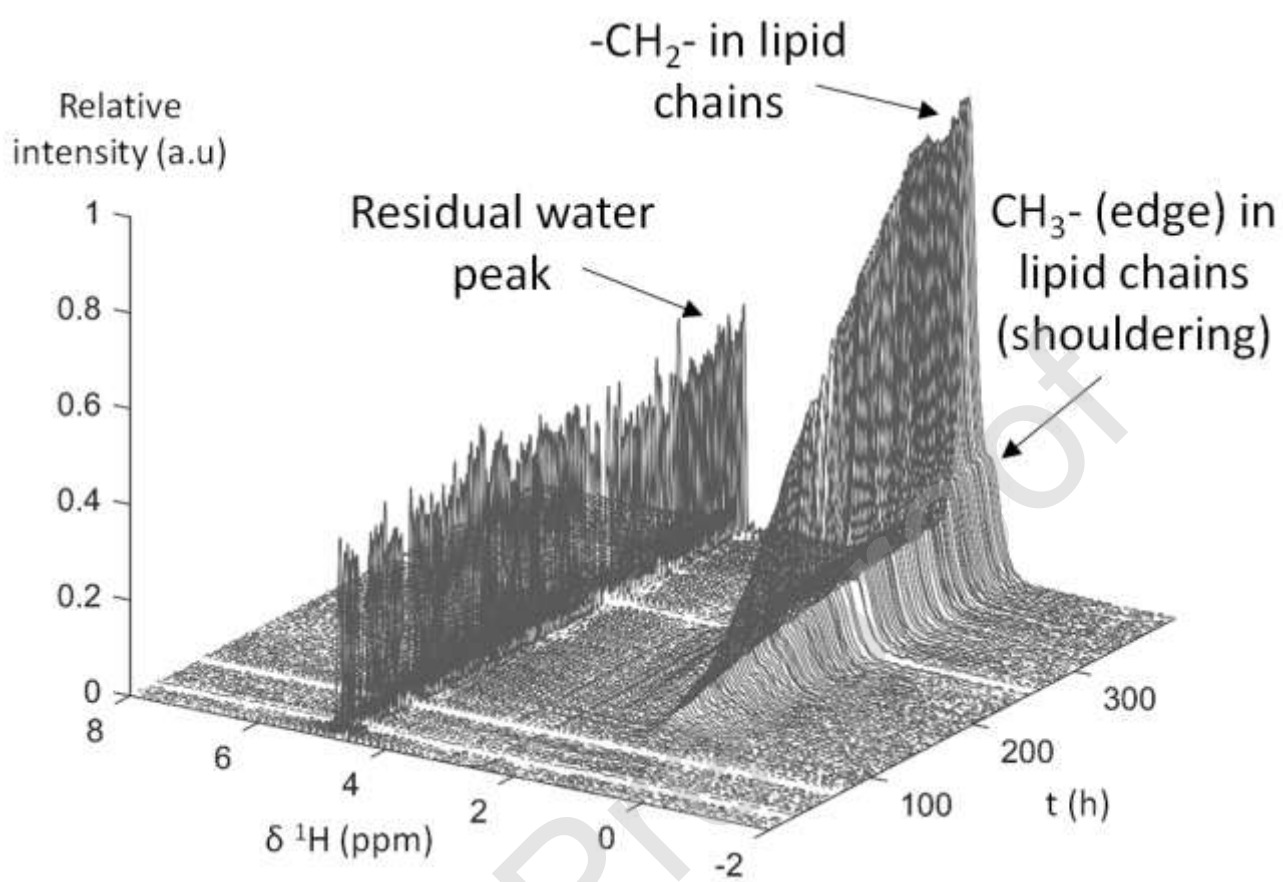


Figure 3

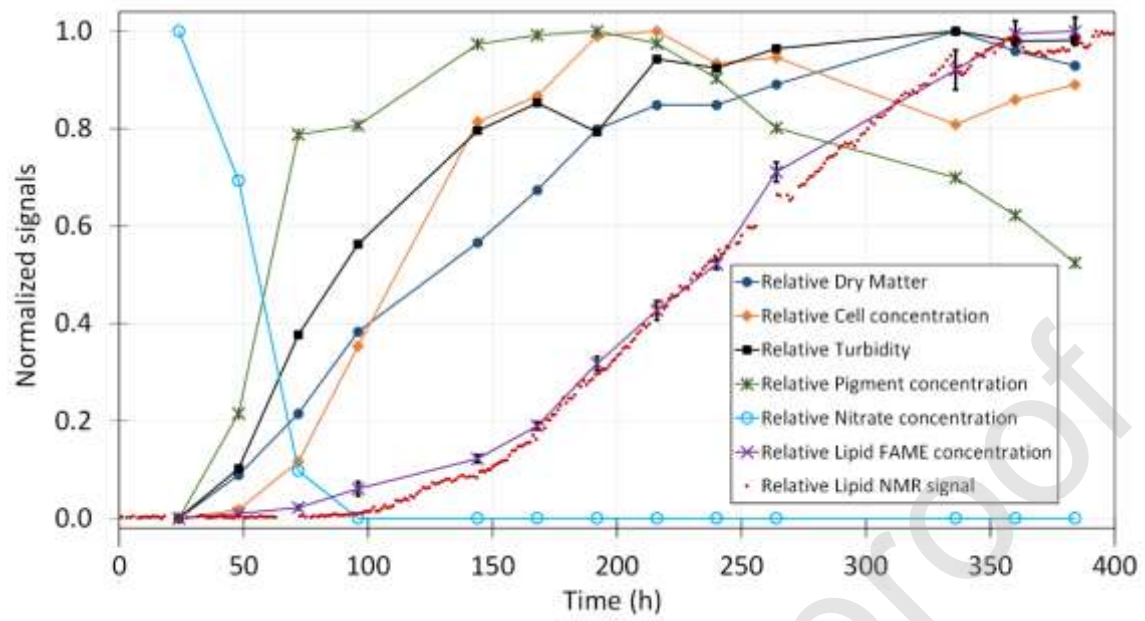


Figure 4

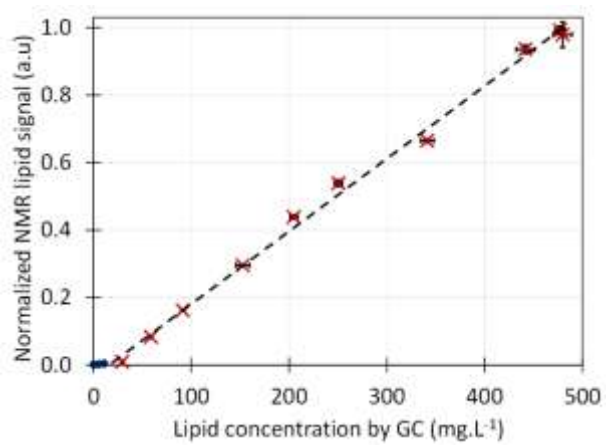
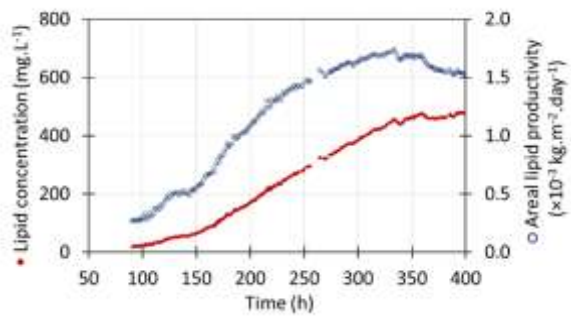
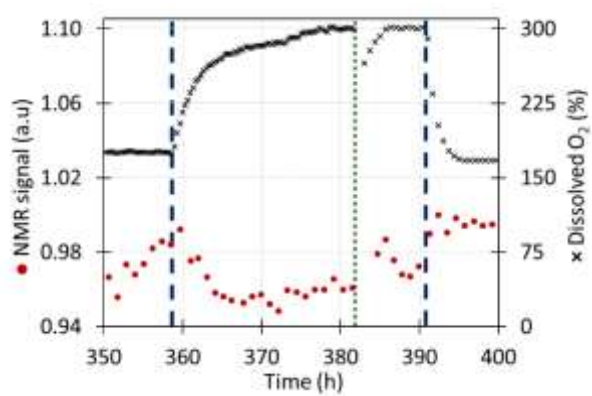


Figure 5



Journal Pre-proof

Figure 6

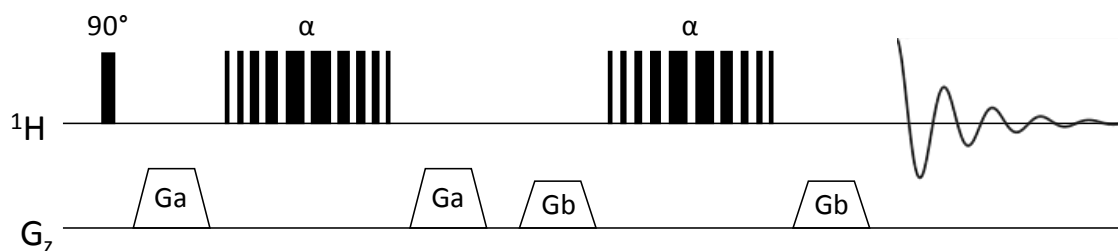


Supplementary Material

Table S1. Bold Basal Medium composition (used for the inoculum)

Compound	Molar concentration (mol.L ⁻¹)
NaHCO ₃	1.50 × 10 ⁻²
NaNO ₃	1.76 × 10 ⁻²
MgSO ₄ ·7H ₂ O	9.13 × 10 ⁻⁴
CaCl ₂ ·2H ₂ O	1.70 × 10 ⁻⁴
EDTANa ₂ ·2H ₂ O	1.34 × 10 ⁻⁴
FeSO ₄ ·7H ₂ O	5.04 × 10 ⁻⁵
K ₂ HPO ₄	8.61 × 10 ⁻⁴
KH ₂ PO ₄	9.04 × 10 ⁻⁴
ZnCl ₂	7.70 × 10 ⁻⁷
Co(NO ₃) ₂ ·6H ₂ O	1.51 × 10 ⁻⁷
CuSO ₄ ·5H ₂ O	4.97 × 10 ⁻⁷
H ₃ BO ₃	4.63 × 10 ⁻⁵
MnSO ₄ ·H ₂ O	9.17 × 10 ⁻⁶
Na ₂ MoO ₄ ·2H ₂ O	1.07 × 10 ⁻⁶

Figure S2. Pulse sequence parameters



W5 pulse sequence. The duration of the hard 90° pulse was tuned at $11.3 \mu\text{s}$ with a power of 0 dB. The α -labelled block was a hard pulse train with the following angle composition: $7.8^\circ - 18.5^\circ - 37.2^\circ - 70^\circ - 134.2^\circ - 134.2^\circ - 70^\circ - 37.2^\circ - 18.5^\circ - 7.8^\circ$. Inside the α pulse train, an inter pulse delay was applied on both sides of each pulse, this delay was optimized at $375 \mu\text{s}$ so the sidebands occur at 36 and -26 ppm being outside of the proton chemical shift range. Gradients had trapezoidal shapes and were applied for $800 \mu\text{s}$ with a power of 42 % (Ga) and 33 % (Gb) of the maximum strength. (20 G.cm^{-1}).

Table S3. Numerical results of various analyses on the photobioreactor cultivation

Time (h)	24	48	72	96	144	168	192	216	240	264	336	360	384
Dry Weight concentration (g.L⁻¹)	0.01	0.16	0.37	0.64	0.94	1.12	1.33	1.41	1.41	1.48	1.66	1.59	1.54
FAME content by GC (% of DW)	3.0	3.2	3.0	4.6	6.2	8.1	11.5	14.5	17.8	23.1	26.6	29.9	31.1
Lipid concentration (mg.L⁻¹)	0.3	5.0	10.9	29.4	59.0	91.3	153	205	251	341	442	477	480
Lipid concentration RMS* (%)	31	18	17	24	6.4	4.2	4.4	4.6	2.0	2.8	4.4	2.7	2.9
Normalized NMR signal (a.u)	0.002	0.003	0.005	0.010	0.084	0.162	0.295	0.439	0.539	0.665	0.936	0.992	0.979
Cell concentration (cells.mL⁻¹ x 10⁶)	1.9	3.7	13	36	81	87	99	100	93	94	81	86	89
Turbidity (/)	0.10	0.25	0.64	0.90	1.23	1.31	1.23	1.44	1.41	1.47	1.52	1.49	1.49
Pigment concentration (µg.mL⁻¹)	1.0	6.2	20.1	20.6	24.6	25.1	25.3	24.6	22.9	20.4	18.0	16.1	13.7
Nitrate concentration (mg.L⁻¹)	210	146	21	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

* RMS: relative mean deviation