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Benchtop flow NMR spectroscopy as an online device for the in vivo monitoring of lipid accumulation in microalgae

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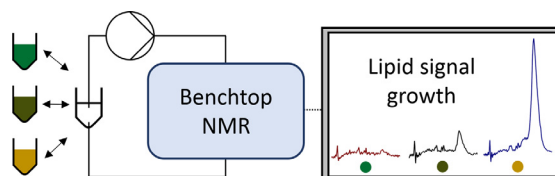
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Graphical abstract

Benchtop flow NMR spectroscopy as an online device for the *in vivo* monitoring of lipid accumulation in microalgae

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Highlights

Benchtop flow NMR spectroscopy as an online device for the *in vivo* monitoring of lipid accumulation in microalgae

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Dylan Bouillaud^{a,b}, Vladimir Heredia^b, Thomas Castaing-Cordier^a, Delphine Drouin^b, Benoît Charrier^a, Olivier Gonçalves^b, Jonathan Farjon^{a,*}, Patrick Giraudeau^{a,c}

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- Benchtop NMR spectroscopy was successfully employed as online *in-vivo* lipid sensor
- Benchtop NMR device can be coupled to microalgae culture bioprocesses
- Benchtop NMR system could be used for control command of microalgae bioprocesses

 Supplementary material



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2 Benchtop flow NMR spectroscopy as an online device for the *in vivo* monitoring of lipid accumulation in microalgae

Q4 Q3 Dylan Bouillaud^{a,b}, Vladimir Heredia^b, Thomas Castaing-Cordier^a, Delphine Drouin^b, Benoît Charrier^a, Olivier Gonçalves^b, Jonathan Farjon^{a,*}, Patrick Giraudeau^{a,c}

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A B S T R A C T

The sustainable production of lipids by microalgae is widely developed among the bioprocess community targeting various applications such as feed, food, health or bioenergy. The cultivation of microalgae needs dedicated systems with the optimal illumination geometry. Performing non-invasive online analyses on these bioprocesses is limited to few analytical techniques, often based on optical properties and can however rarely be related to intracellular products. The real-time knowledge of the lipids accumulation in microalgae is –in this case– not possible. In this article, the proof-of-concept that the recent benchtop NMR spectroscopy device can be used for the non-invasive and selective detection of lipids inside microalgae cells is carried out. Three cultures of *Nannochloropsis gaditana* were analyzed in flow conditions. The relative quantitative feature is confirmed by the correlation with a reference technique classically used for lipid analysis, *i.e.* the FAME (Fatty Acid Methyl Ester) profiling by gas chromatography.

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41 1. Introduction

42 For several years, microalgae have aroused a certain interest since
 43 they are able to produce a large amount of biomass, including various
 44 valuable compounds. The cultivation of microalgae turns out to be a sus-
 45 tainable alternative to terrestrial crops. Among these compounds of in-
 46 terest, lipids are involved in several applications, especially feed [1] (as
 47 food supplements, for food-processing industry or animal farming) and
 48 for biofuel production [2]. Some species of microalgae are able to pro-
 49 duce a great amount of reserve lipids, mainly due to the metabolic
 50 shift occurring during nitrogen starvation or limitation. However, the
 51 complexity of the operational protocols, imply to develop an accurate
 52 monitoring of the synthesis of the targeted lipids to ensure the success-
 53 ful conduct of the bioprocess.

54 The main bottleneck consists finally in accessing to the intra-cellular
 55 lipid content both quantitatively and qualitatively. In order to analyze
 56 the microalgae lipids, the conventional method first consists in an ex-
 57 traction step with –often customized– Bligh and Dyer [3] or Folch [4]
 58 methods. Then it is followed by a simple gravimetric analysis or a
 59 more-informative FAME (Fatty Acid Methyl Ester) analysis using GC
 60 (Gas Chromatography) after transesterification. The latest approach
 61 can be considered as being a flagship method since it provides both

qualitative and quantitative results, needing limited amount of sample. 62
 Moreover, the composition in fatty chains provides insights about the 63
 nutritional properties of the lipid extract as determining also the quality 64
 of the oil for biodiesel application [5]. Other analytical techniques, often 65
 depending on laboratory specialty and equipment, can be used in place 66
 or in addition to GC. Briefly, lipid classes can be analyzed with different 67
 combined approaches: with or without a preparative separation or ex- 68
 traction step, followed by analysis on a support such as TLC (Thin 69
 Layer Chromatography) [6] or HPLC (High Performance Liquid Chroma- 70
 tography) [7], sometimes improved with a MS (Mass Spectrometry) 71
 coupling [8]. In any way, the chosen analysis needs several offline 72
 steps that are both time and sample-consuming and can only provide 73
 a result several hours after sampling –in the best case scenario. There- 74
 fore, such methods do not allow real-time monitoring. Other techniques 75
 without lipid extraction are also used: Nile red staining [9], flow cytom- 76
 etry [10], TD-NMR (Time-Domain Nuclear Magnetic Resonance) [11], 77
 Infrared spectroscopy [12] or Raman spectroscopy [13]. These techni- 78
 ques have their own advantages and limitations but –to the best of 79
 our knowledge– none of them has been used for online non-invasive 80
 monitoring of lipids in living microalgae cells. 81

In this context, NMR spectroscopy is a good candidate for such analy- 82
 sis since this analytical technique provides accurate quantitative infor- 83
 mation in a non-destructive way and is compatible with flow 84
 measurements. The use of NMR on whole microalgae cells has already 85
 been studied in both solid- and/or liquid-state NMR [14,15]. The in- 86

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cell detection of lipids is feasible, but conventional NMR suffers from practical incompatibilities since high-field spectrometers are expensive to purchase and maintain but are also massive, unmovable and can only be coupled to customized culture systems [16]. In order to improve the accessibility and cost of NMR, benchtop NMR spectroscopy devices have emerged recently. Based on permanent magnets, they are cryogen-free and can easily be coupled with all kinds of online set ups, thus being suitable for reaction and process monitoring. The main drawback is the drastic decrease of the magnetic field (compared to conventional spectrometers), which is –on current devices– in the 1–2 Tesla range. This implies a loss of both resolution and sensitivity. In addition to this first limitation, obtaining NMR spectra on living microalgae in their cultivation medium requires dealing with the predominant water signal, which covers all the other peaks.

In this article, the proof-of-concept that lipids in entire cells can be detected by online NMR spectroscopy and in their real culture medium is performed. Methodological NMR developments were first carried out to efficiently remove the water peak and to enhance the lipid signal detection. The spectra were acquired on whole microalgae for three cultures of *Nannochloropsis gaditana*, a marine microalga of interest, in three different physiological stages with different amount of accumulated neutral lipids. Through the analysis of these three cultures, the ability of benchtop NMR to monitor lipid accumulation in living microalgae cells without disturbing the bioprocess was carried out.

2. Material and methods

2.1. Microalgae cultures

Three independent cultures of *Nannochloropsis gaditana* CCMP527 were analyzed in this work: one reference culture (NG1) and two nitrogen-starved cultures (NG2 and NG3) with different starvation stages in order to obtain different intracellular lipid concentrations. 100 mL of three cultures from three independent photobioreactors were collected simultaneously. The microalgae were grown following the recommendation established by Taleb et al. [17].

2.2. Dry weight and cell number determination

In order to characterize the three cultures, their dry weight and cell concentration were measured. 10 mL from each culture were filtered through a dried glass-fiber filter (Whatman GF/F). The filters were dried 24 h in an oven at 105 °C before weighing. Filters were then weighed before and after microalgae filtration to determine the biomass dry weight. Each culture was measured in triplicate. The cell number of each culture was then determined using a Malassez counting cell under optical microscope.

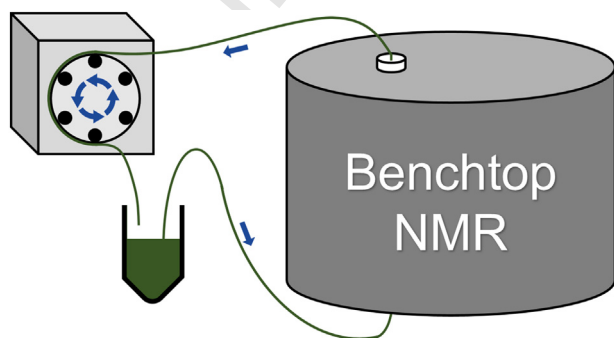


Fig. 1. Experimental setup scheme: use of benchtop NMR on microalgae cultures as an online detector in flow conditions.

2.3. Experimental setup

The experimental setup represented in Fig. 1 did not require any sophisticated equipment, thus making it applicable in a routine context. A small volume of microalgae culture was conveyed with a peristaltic pump through the benchtop spectrometer in a commercial glass flow cell. The total volume of the loop –ca. 5 mL– was negligible in comparison to the typical volume of a microalgae culture (from 500 mL to several liters) so it would not disturb the culture in the study of real-scale bioprocesses. The flow rate was set to 1.0 mL·min⁻¹. As previously shown, this value does not significantly impact the NMR detection since apparent relaxation times are not significantly affected [18], while it ensures a good sample homogeneity, ensuring an optimal signal sensitivity during the measurement.

2.4. Benchtop NMR

The benchtop NMR spectrometer was a Spinsolve ¹H/¹⁹F/¹³C from Magritek (Germany) delivering a 1.0 T magnetic field for a proton frequency of 43.5 MHz. As mentioned above, the device was equipped with a gradient coil along the B₀ direction. No deuterated substance was needed thanks to an external fluorine lock system. The spectra were acquired using the SpinsolveExpert software. For each spectrum, 3600 scans were accumulated with a repetition time of 1 s to obtain a one-hour spectrum, which was chosen as a good compromise between the sensitivity of detection (NMR scan accumulation) and the measurement rate, to obtain an appropriate sampling of the kinetic data. The experiment duration for one spectrum was set to be compatible with the kinetics of typical microalgae cultures, which lasts a few weeks. For every recorded free induction decay (FID), 4096 points were sampled with a dwell time of 50 μs resulting in a 204.8 ms acquisition time. The pulse sequences used in order to remove the water peak are assessed in the dedicated section below (Section 3.1). The carrier frequency was adjusted to the water frequency before each set of spectra. Detailed pulse sequence parameters are described in Supplementary material (Fig. S1.A). Five spectra were acquired on each culture to evaluate the analytical repeatability. Spectra were manually phased and integrated with the MestReNova software.

2.5. High-field NMR

High-field NMR experiments were carried out on a 16.4 T Bruker Avance-III HD spectrometer operating at a ¹H frequency of 700.13 MHz, equipped with an inverse ¹H/¹³C/¹⁵N/²H cryogenically cooled probe. 10% D₂O was added to each culture as a lock reference. High-Field NMR was used for the sake of comparison and also for control, especially to check that no signal overlapped with the peak of interest. High-Field NMR spectra are available in Supplementary material (Fig. S.2).

3. Results and discussion

3.1. Optimization of the water peak suppression

Performing NMR spectroscopy on microalgae samples (i.e., without deuterated water) with a classical one-pulse acquisition would lead to a unique and intense water peak, since water protons are ubiquitous compared to protons from lipids of interest whose concentration is much lower (see the top spectrum of Fig. 2). Thanks to advanced pulse sequences, suppressing the solvent peak in order to get a ¹H spectrum in non-deuterated solvents is well-known in the high-field NMR community [19]. The majority of these pulse sequences uses magnetic field gradients which requires therefore a gradient coil in the hardware. This equipment has been recently implemented on commercial benchtop NMR devices [18] and is essential for this work.

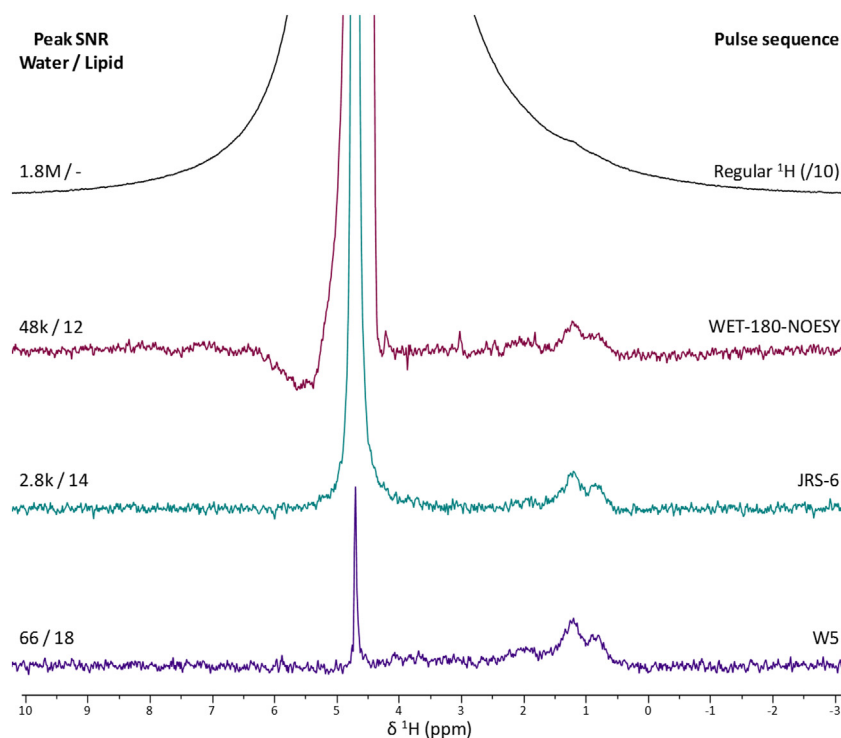


Fig. 2. Evaluation of water suppression pulse sequences on a benchtop spectrometer, on non-starved concentrated *Parachlorella kessleri*. From top to bottom: regular ^1H spectrum (the water peak is so intense that the spectrum intensity has been divided by 10); the WET-180-NOESY spectrum; the JRS-6 spectrum and the W5 spectrum. A manual baseline correction has been applied for the four spectra. Residual water peaks are visible at 4.7 ppm. The efficiency of the water peak reduction was taken into account as the main criterion for choosing the pulse sequence.

186 Several strategies exist to get rid of the solvent peak, which either
 187 rely on a selective suppression of the corresponding resonance or on
 188 the selective excitation of all the resonances except the solvent one.
 189 The choice among these techniques relies on two main criteria: i) the ef-
 190 ficiency of the solvent peak reduction ii) the selectivity of the suppres-
 191 sion. Indeed, a given bandwidth is impacted by the pulse sequence
 192 and this suppressed bandwidth should be narrow enough to prevent
 193 the removing of other peaks close to the solvent one. In concrete
 194 terms, each method is a compromise between these two criteria. There-
 195 fore, a preliminary work to optimize the water peak suppression was
 196 carried out on concentrated microalgae after centrifugation to obtain
 197 microalgae spectra in a reasonable time. These preliminary experiments
 198 were performed on a non-starved oleo-microalgae: *Parachlorella*
 199 *kessleri* UTEX2229. Fig. 2 presents four spectra acquired on the same
 200 concentrated microalgae sample in flow conditions: the classical ^1H
 201 spectrum which leads to a unique intense peak, and three different sol-
 202 vent suppression pulse sequences which correspond to three different
 203 approaches for removing the water peak:

204 i) WET-180-NOESY (Water suppression Enhanced through T_1 effects
 205 - 180° pulse - Nuclear Overhauser Effect Spectroscopy), in a previous
 206 work on a similar device [20], this pulse sequence appeared as the opti-
 207 mal solvent suppression pulse sequence for small molecules thanks to a
 208 high selectivity, *i.e.*, a low disturbance of peaks closed to the water one.

209 ii) W5 (WATERGATE-5: WATER suppression by GrAdient-Tailored
 210 Excitation) [21], a well-known suppression pulse sequence for its
 211 great efficiency in removing water peak despite poor selectivity for
 212 water. This pulse sequence has been successfully employed in several
 213 studies on environmental samples [22–24].

214 iii) JRS-6 (Jump-and-Return Sandwiches, with 6 indicating that it is a
 215 6-pulse version of the pulse sequence) a WATERGATE based pulse se-
 216 quence with modulated pulses in order to modulate the suppressed
 217 bandwidth [25]. Other water suppression variants have been tried but
 218 are not shown in this article, since they do not lead to better perfor-
 219 mance. All these pulse sequences were implemented and optimized

on the benchtop spectrometer and their performance was subsequently 220
 221 assessed.

222 The results are shown on Fig. 2. The regular ^1H spectrum highlights
 223 the need to remove the predominant water peak at 4.7 ppm. The
 224 broad peak around 1.2 ppm that can be observed on water-
 225 suppressed spectra corresponds to the microalgae intracellular lipids
 226 (see annotations in the next section) and, thus, is the peak of interest
 227 for this study. The evolution of SNR (Signal to Noise Ratio) of both
 228 water and lipid peaks on the different spectra highlights that a strong
 229 water suppression is critical to obtain an exploitable lipid peak. Due
 230 to the large frequency difference (150 Hz) between the water and lipid
 231 peaks, the selectivity of the water suppression is not critical as it was
 232 the case for small molecules [20]. The W5 pulse sequence leads to opti-
 233 mal results and was chosen for the online monitoring (pulse sequence
 234 parameters in Supplementary Material Fig. S.1).

3.2. ^1H NMR on microalgae: annotations 235

236 The high-field ^1H NMR spectrum of microalgae provides several lipid
 237 signals. Fig. 3 shows three spectra with their assignments, acquired on
 238 starved microalgae studied in this work. As reference, a triglyceride
 239 molecule is represented (Fig. 3.A) with assigned protons. The top spec-
 240 trum (Fig. 3.B) is a lipid extract from NG3 after chloroform-methanol
 241 extraction [26] recorded at 700 MHz. This spectrum is a typically-
 242 shaped lipid spectrum that can be observed on edible oils, for example
 243 [27]. The most intense peak at 1.2 ppm corresponds to all the methylene
 244 protons in saturated parts of fatty chains. Others protons differ in chemi-
 245 cal shift due to unsaturation and/or to the carbonyl group proximity.

246 The second spectrum (Fig. 3.C) was acquired on the same culture
 247 (NG3) with the same spectrometer (700 MHz) but without any extrac-
 248 tion step, *i.e.*, on whole microalgae cells. Lipid peaks can still be observed
 249 but are much broader. This is due to the suspension state of the
 250 microalgae culture: this particular suspension state makes peaks signif-
 251 icantly broader in comparison to spectra of well-solubilized analytes

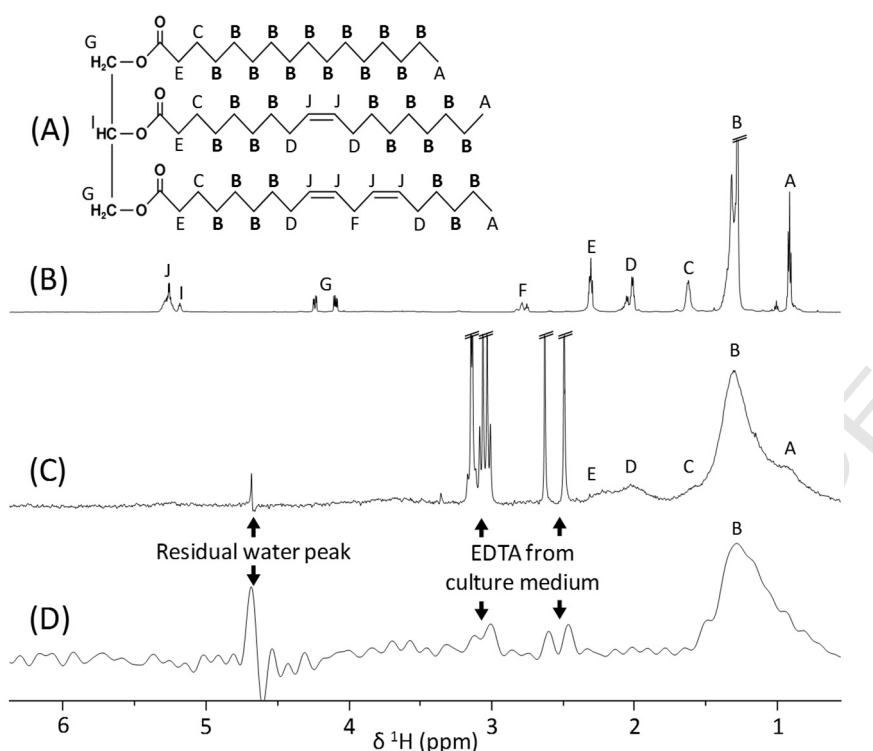


Fig. 3. ^1H spectra from a starved *Nannochloropsis gaditana* culture. (A) is the structure of a triglyceride with the assignment of protons which are visible on the spectra below (B) is a lipid extract from the NG3 culture, dissolved in CDCl_3 and analyzed at 700 MHz. (C) is the raw culture with D_2O for lock reference analyzed at 700 MHz after water peak suppression with W5 pulse sequence. (D) is the raw culture analyzed at 43 MHz after water peak suppression with W5 pulse sequence. The more intense lipid peaks on extract spectrum (B) are detectable on the whole microalgae spectrum at 700 MHz (C). The most intense peak B remains visible at 43 MHz and can be used for the non-invasive monitoring of total lipids.

since solid samples are subjected to anisotropic interactions. Moreover, the cells settle spontaneously at the bottom of the NMR tube in the measurement time, leading to a lack of sample homogeneity. Around 2.5 and 3.0 ppm, sharp peaks correspond to the EDTA (ethylenediaminetetraacetic acid) complex present in the cultivation medium.

The bottom spectrum (Fig. 3.D) is the same culture analyzed with the benchtop spectrometer at 43 MHz in flow conditions, which actually helps to keep the sample homogeneous all over the course of the analysis, which was not the case with high field spectrometer. Despite the loss of magnetic field, the major lipid peak around 1.2 ppm remains clearly visible, thus, confirming that benchtop NMR is able to selectively detect lipids inside the living microalgae cells. It should be kept in mind that the source of this signal corresponds to all the protons in the saturated parts of the lipid chains (labeled B in Fig. 3.A). NMR provides a quantitative signal on these protons that corresponds to the majority of the lipid protons. So it can be reasonably related to the total lipid amount biosynthesized by the microalgae.

3.3. Assessment of the usage of the benchtop NMR spectroscopy as a non-invasive lipid sensor

As mentioned above, three cultures corresponding to different levels of nitrogen starvation (NG1, NG2 and NG3) were studied in flow conditions with the benchtop spectrometer. One spectrum over five replicates is shown on Fig. 4. For the non-starved culture NG1, the lipid peak is very weak since the microalgae have not started to accumulate them yet. For NG2 and NG3, a much stronger lipid signal is observed, with SNR of 40 and 170 for NG2 and NG3, respectively. This first result indicates that NG3 have accumulated more lipids than NG2. All the lipid peaks were integrated and the resulting quantitative results are presented in Table 1, in addition to the dry weight and cell counting measurements. The repeatability assessment (RSD over 5 measurements) gives 34, 7 and 4% for NG1, NG2 and NG3 respectively. The bad repeatability for NG1 was expected since the peak presented a

very low SNR. However NG2 and NG3 spectra show that the repeatability of the method is fully compatible with real culture conditions.

Regarding the accuracy, the three NMR integrals were compared with the results of FAME analysis after lipid extraction and derivatization. The whole FAME analysis protocol is described in Supplementary Material Section 3. This analysis provides a good estimation of total lipid and is used as a reference technique. Both relative signals have been normalized to the dry weight for sake of comparison. The coefficient of determination after linear regression is higher than 0.98, suggesting a very good linear correlation between the NMR lipid signal and the total lipid concentration as estimated by the reference quantification approach. Indeed, although the dry weight and cell counting results were quite different for the three cultures, benchtop NMR provides a total lipid signal independent of the cell concentration. The relative quantification is similar to the one obtained with FAME analysis -without the need for extraction and derivatization- thus showing the potential of benchtop NMR to quantitatively monitor the accumulation of lipids in real-time, in a repeatable and non-destructive way.

3.4. Perspectives

This work paves the way to the application of the use of benchtop NMR spectroscopy for the online quantitative measurements of total lipid amounts in fresh microalgae cells. The next step of this work would be the coupling of the benchtop NMR apparatus to a working photobioreactor in which lipid accumulation with nitrogen starvation is operated on the grown microalgae. Since the development of benchtop spectroscopy is still in its early days, future hardware improvements -particularly in terms of magnetic field strength- will certainly help reaching such a goal in the near future. While the current method can only monitor relative concentration variations due to the impossibility to add an internal reference in the culture medium while remaining non-invasive, the implementation of an electronic reference such as ERETIC (Electronic REference To access *In vivo* Concentrations) [28] is

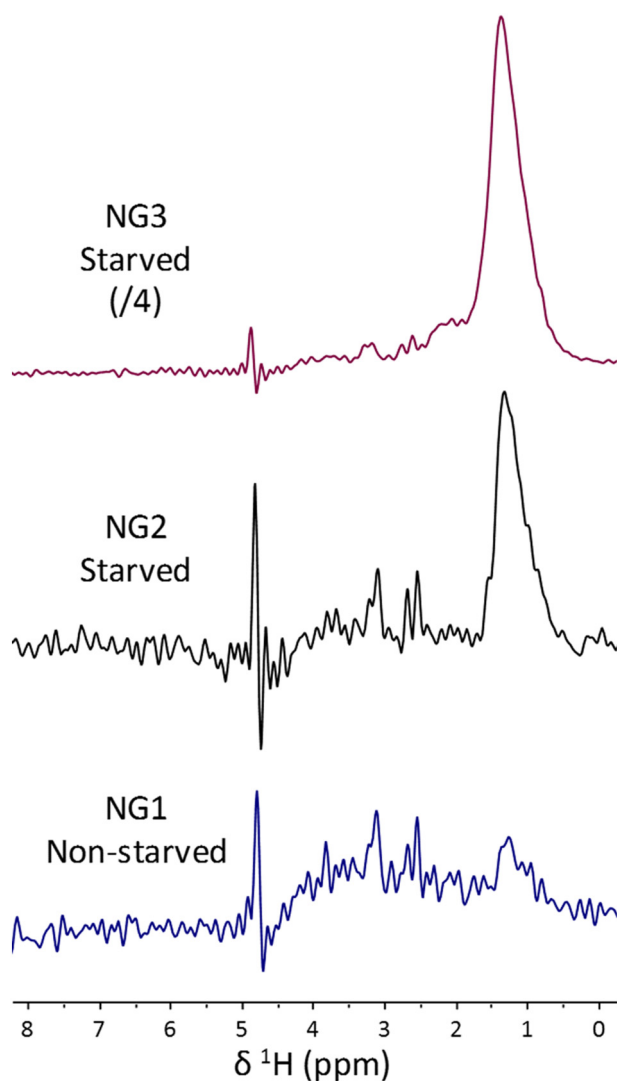


Fig. 4. Online ^1H NMR spectra recorded on whole microalgae with the W5 pulse sequence, in flow conditions on a benchtop spectrometer. A residual water peak is present at 4.7 ppm. EDTA peaks from the culture medium are visible between 2.5 and 3.2 ppm. Three states of lipid accumulation are clearly distinguishable from the intensity of the lipid peak at 1.2 ppm. The NG3 spectrum intensity was divided by 4.

on this kind of hardware would provide a way to measure absolute concentrations. Finally, this scaling down of NMR devices could be pushed forward even more with the further miniaturization of NMR sensors [29].

4. Conclusion

The proof of concept that benchtop NMR spectroscopy can be used as an online non-invasive sensor for the real-time monitoring of lipids inside microalgae cells was performed. The benchtop size of the device

Table 1

Numerical results on NG1, NG2 and NG3 cultures; lipid peak integration from NMR spectra, total FAME from GC, dry weight and cell counting.

Culture	Lipid peak integral average (a.u)	NMR RSD (%)	GC FAME content (%) of DW)	Dry Weight ($\text{g}\cdot\text{L}^{-1}$)	Cell concentration (per mL)
NG1	$7.3 \cdot 10^{-4}$	34	15.4	1.14	$9.0 \cdot 10^8$
NG2	$2.3 \cdot 10^{-3}$	6.9	39.0	0.63	$1.3 \cdot 10^9$
NG3	$1.2 \cdot 10^{-2}$	4.3	52.6	2.00	$9.7 \cdot 10^8$

and the negligible loop volume make the analysis non-invasive regarding the bioprocess. Through the optimized W5 pulse sequence, a lipid peak is detectable and integrable for the online monitoring of the total lipids. Despite the lack of sensitivity measured for the non-starved cultures, the analytical performance assessment on three microalgae cultures shows that benchtop NMR should be reasonably sensitive to measure the relative evolution of lipid concentration.

Declaration competing interest

The authors declare that there is no conflict of interest.

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Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Contributions

DB performed NMR experiments on high and low field instruments, analyzed data and wrote the article. VH performed microalgae cultures in photobioreactors. TCC implemented and optimized solvent suppression methods on the benchtop spectrometer. BC was in charge of managing flow tools. DD performed gas chromatography analyses. 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 to this article can be found online at <https://doi.org/10.1016/j.algal.2019.101624>.

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