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# Optimization of continuous TAG production by *Nannochloropsis gaditana* in solar-nitrogen-limited culture

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## Abstract

Nitrogen limitation and changing solar conditions are both known to affect triacylglycerol (TAG) production in microalgae. This study investigates the optimization of TAG production with a continuous nitrogen-limited culture of *Nannochloropsis gaditana* in simulated day–night cycles (DNc). The effect of DNc was first investigated in nitrogen-deprived condition (i.e., batch culture), emphasizing a significant change in mechanical resistance of the strain during the night. The concept of released TAG, which shows how much of the TAG produced is actually recovered in the downstream stages, that is, after cell disruption, was shown here of interest. For a maximum released TAG, the optimum harvesting time was suggested as being 4 h into the night period, which minimizes the losses due to a too great cell mechanical resistance. The protocol for continuous nitrogen-limited culture was then optimized, and a continuous nitrogen addition was compared to a pulsed-addition. For the latter, nitrogen was supplied in a single pulse at the beginning of the light periods, while the bulk medium was supplied separately at a slow but constant dilution rate of  $0.005 \text{ h}^{-1}$ . The pulse dose was calculated after the study of nitrogen consumption and TAG production/consumption during the DNc. The estimated released TAG for the pulsed-addition of  $1.4 \times 10^{-3} \text{ kg/m}^2\text{d}$  found significantly higher than the one achieved in batch culture ( $0.3 \times 10^{-3} \text{ kg/m}^2\text{d}$ ) but lower than for continuous nitrogen addition which obtained the highest released TAG of  $3 \times 10^{-3} \text{ kg/m}^2\text{d}$ .

## KEYWORDS

biodiesel, cells mechanical resistance, day–night cycles, lipids, nitrogen limitation

## 1 | INTRODUCTION

The current climate change crisis and the increasing demand for energy in an ever-growing society are the main drivers for continuing the research about biofuels (Cavicchioli et al., 2019; WMO, 2019; Zhu et al., 2017). Among these fuels, biodiesel produced from microalgae may contribute to replacing oil-based fuels in a near future, but only if economic policies and manufacturing technologies are improved (Baudry et al., 2017; Lee, 2011). Many microalgae species have been studied for a biodiesel application (Mutanda

et al., 2011; Taleb et al., 2015). In particular *Nannochloropsis gaditana* is perceived to be a promising TAG producer (Bouillaud et al., 2019; Lubian, 1982; Matos et al., 2017; Mitra et al., 2015). Large-scale production processes require outdoor cultivation facilities that use solar energy to produce the triacylglycerol (TAG) molecules that will be recovered and converted into biodiesel. Cultivation in photobioreactors (PBR) that use sunlight from day–night cycles (DNc), combined with nitrogen limitation or depletion protocols, is considered a good approach (Chisti, 2007; Rodolfi et al., 2009; San Pedro et al., 2013, 2014).

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Different initial levels of nitrate concentration ( $\text{NO}_3^-$ ) during the culture lead to different values of biomass production and TAG content in *Nannochloropsis* (Taleb et al., 2015) and other species (Kandilian et al., 2019). Also, initial  $\text{NO}_3^-$  concentration can induce complex metabolic responses (Alboresi et al., 2016; Dortch, 1982; Van Vooren et al., 2012). In this regard, Simionato et al. (2013) and Janssen, Wijffels et al. (2019) discovered that membranes are reorganized during nitrogen depletion, which throws light on the de novo lipids synthesis in *N. gaditana*.

In outdoor conditions, sunlight and other varying conditions may also induce metabolic responses. Khalid et al. (2019) showed that light intensity and photoperiods affect the nutrient uptake rate in *Chlorella sorokiniana*. Similarly, Kandilian et al. (2014) showed that large accumulations of fatty acids and TAG in *Nannochloropsis oculata* are strongly dependent on the photon absorption rate, and not just on nitrogen depletion. However, high values of light and temperature can also inhibit microalgal growth despite a higher TAG accumulation (Alboresi et al., 2016; Figueroa et al., 1997). In addition, losses in TAG and biomass production were linked to respiration during night periods, causing the reduction of accumulated TAG (Bonfond et al., 2016; Edmundson & Huesemann, 2015; Grobbelaar & Soeder, 1985; Lacour et al., 2012; Richmond, 1996).

Additionally, some microalgae species show issues related to metabolite extraction, which is related to cell wall resistance (Safi et al., 2013). *Nannochloropsis gaditana* has been described as particularly resistant to the TAG wet-extraction process when cultivated under nitrogen depletion. Angles et al. (2017) and Scholz et al. (2014) observed a decrease in cell disruption rate when the cells are nitrogen depleted.

Currently, the simplest approach for producing TAG molecules is by combining two culture stages in continuous light: one culture in optimum growing conditions for biomass production only, followed by a batch culture in nitrogen depletion (sudden or progressive). Van Vooren et al. (2012) achieved surface TAG productivity of up to 0.0036 and 0.0028  $\text{kg}/\text{m}^2\text{d}$  with *N. oculata* in sudden starvation and progressive starvation batches respectively. A semicontinuous culture mode can also be used for the biomass production stage as shown by Taleb et al. (2015). Under this system a volumetric TAG productivity of around 0.013  $\text{kg}/\text{m}^3\text{d}$  was obtained with *N. gaditana*.

Alongside the two-stage approach, it is also possible to produce TAG molecules in a single operation, by using either batch or semicontinuous mode (Benvenuti, Bosma, et al., 2016; Benvenuti, Lamers, et al., 2016). Also continuous production in DnC, was approached by Bonfond et al. (2016) using *Dunaliella salina*. This approach enabled repeated daily variations, very similar to a steady state in classic continuous mode. As expected, the TAG reserves were consumed in the dark period (when there is no source of energy), and renewed in the light period.

Based on all the previous considerations, establishing a protocol to produce TAG-rich biomass in continuous mode during DnC is not straightforward. The specific growth rate is influenced by nitrogen supply/uptake and time-varying light absorption, both of which are related to the nitrogen-source availability due to the decrease in

pigment concentration when cells are exposed to nitrogen limitation (Kandilian et al., 2014, 2019). Such protocol would require optimizing the nitrogen supply/uptake, and the harvesting periodicity, both as a function of DnC. In this regard, to obtain a steady state for continuous harvesting under DnC, the biomass produced during the light period (daytime) should be equal to the amount of biomass harvested and consumed during the dark period (night). Therefore, the dilution rate  $D$ , must be (i) low enough to allow cell growth during day period and (ii) not too high to wash out the culture, as it will be combined with the loss of biomass during the night period.

Establishing a continuous protocol with nitrogen limitation and DnC is not straightforward; the specific growth rate is influenced by nitrogen supply/uptake and time-varying light absorption, both of which are related to the nitrogen-source availability due to the decrease in pigment concentration when cells are exposed to nitrogen limitation (Kandilian et al., 2014, 2019).

This study analyses the potential of such protocol for TAG production using the marine microalga *N. gaditana*. Multiple aspects are expected to change during DnC, which could impact biomass growth and concentration, TAG content, cell wall composition and nitrogen uptake. The present work considers these variations for recovering TAG molecules during a wet-pathway process, as suggested by Heredia et al. (2021)

The approach is divided into two main experiment sets: (I) analysis of the released TAG value (after cell disruption) produced in sudden starvation and under DnC, to investigate whether the cycles have any effect on cell mechanical resistance; (II) establishment of a continuous TAG production protocol in nitrogen-limited culture conditions and DnC, taking advantage of the time-varying physiological changes identified in the experiment set (I), in an attempt to optimize TAG production and recovery via a wet-pathway process.

## 2 | MATERIALS AND METHODS

### 2.1 | Culture medium

*Nannochloropsis gaditana* CCMP527 (NCMA) was cultivated using artificial sea water (ASW) (Berges et al., 2001) as the main medium, with CONWAY solution for nutrient enrichment. ASW is composed as follow (mM): NaCl, 248;  $\text{Na}_2\text{SO}_4$ , 17.1; KCl, 5.49;  $\text{H}_3\text{BO}_3$ , 0.259; NaF, 0.045;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 32.24;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.626; KBr, 0.497;  $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.056;  $\text{NaHCO}_3$ , 1.42.

Originally, CONWAY solution uses 10.6 mM of  $\text{NaNO}_3$  as the nitrogen source. For the media prepared in this study under different  $\text{NO}_3^-$  concentrations, CONWAY solution was still used but the relative amount of  $\text{NaNO}_3$  was varied. Hereafter, these values will be expressed as a percentage of the original formulation (100% $\text{NO}_3$  for 10.6 mM  $\text{NaNO}_3$ ) to represent the depletion level. For example, depending on the experiment, systems were prepared with media solutions at 200%, 100%, 56%, 46%, 29% or 0% $\text{NO}_3$  (21.2, 10.6, 5.94, 4.88, 3.07 and 0 mM of  $\text{NO}_3^-$ , respectively). In addition, the culture at 200% $\text{NO}_3$  is referred to as *replete* (its purpose to represent optimal

growth without nitrogen limitation). Likewise, cultures referred to as *depleted* had no nitrogen source added (0% $\text{NO}_3$ ).

For the pulsed-addition of  $\text{NO}_3^-$ , an independent solution at 126 mM of  $\text{NaNO}_3$  was prepared and added separately from the rest of the culture bulk medium.

## 2.2 | Indoor reactors

For the study on depleted culture in simulated DNC, a 170 L (5 cm thick) flat-panel airlift PBR (HECtor, Figure 1a) was operated in batch mode. The pH was automatically regulated at 8 by automatic 98%  $\text{CO}_2$  (gas) injection. The temperature of the culture was regulated to 23°C. More details on the PBR are available in Pruvost, Van Vooren et al. (2011).

The indoor experiments on establishing continuous TAG production in nitrogen depletion and DNC were carried out in 1 L (3 cm thick) flat-panel airlift PBRs (Figure 1b). More details are given in Pruvost et al. (2009). The temperature was controlled at room temperature, 23°C, and the pH was set at 8 by automatic 98%  $\text{CO}_2$  (gas) injection. Following sterilization, the PBR was either filled with the corresponding depleted medium for batch culture or adapted for operation in continuous mode using a peristaltic pump for feeding (Reglo ICC). Then cultures were inoculated (at 10%v/v and 0.2 kg/m<sup>3</sup> for batch the batch culture) using *N. gaditana* seed culture in exponential growth phase.

## 2.3 | Simulated DNC

Solar cycle simulations for the indoor PBR system were emitted by a LED PAR-light panel (2 × 80 white LED, by Global Process Concept, France for the HECtor system; and 70 white LED by Bio-Concept Tech., for the 1 L PBR). Calibration between light received by the PBR and LED power supply was established using a quantum sensor for the PAR spectrum (LI-190-SA sensor with LI-250-A meter, LI-COR) placed on the inner wall of the PBR. Once calibration was established, DNC were programmed (Arduino) by varying the light supply to the

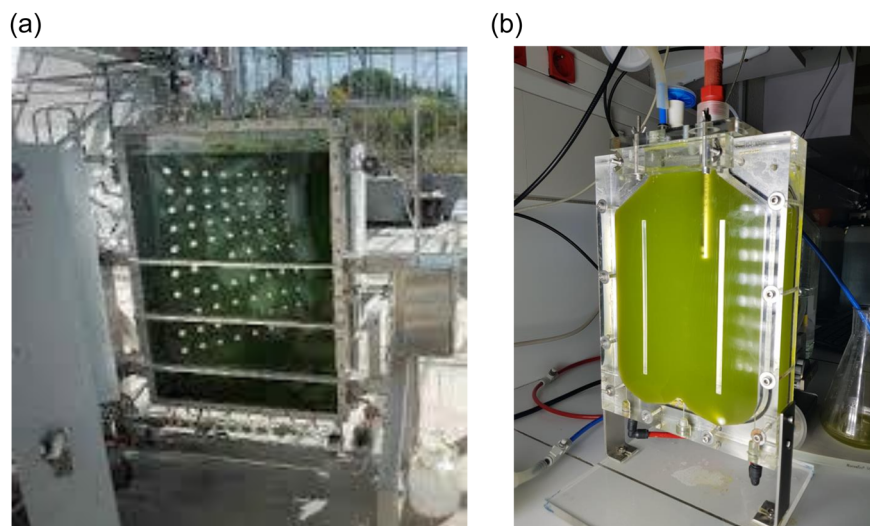
culture every 15 min in a 24 h loop. Values were obtained for the average photosynthetically active radiation ( $\text{PFD}_{\text{PAR}}$ ,  $\mu\text{mol}/\text{m}^2\text{s}$ ) in an entire year at Saint-Nazaire in France from a meteorological database (Meteonorm). The final DNC averaged 269  $\mu\text{mol}/\text{m}^2\text{s}$ , a maximum of 847  $\mu\text{mol}/\text{m}^2\text{s}$  at noon, and a day-to-night ratio of 16:8 h. For convenience, DNC started at 00:00 h as the start of the light period, and ended at 23:59 as the last hour of the night period.

## 2.4 | Cell disruption rate

A 30 ml sample at a biomass concentration of around 1 kg/m<sup>3</sup> (adjusted with phosphate buffer saline [PBS]) was passed through a high-pressure homogenization device (TS5, Constant Systems Limited) at 1750 bar. Cells were counted via image analysis before and after the disruption. For each sample, 40 pictures were taken in a Malassez chamber using an optical microscope attached to a camera (Axio MRC Cam at Axio Scope A1 microscope, Carl Zeiss). The samples were prepared according to a Zinkoné et al. (2018) procedure. The cell surfaces for each picture were calculated using image pretreatment software (ImageJ v.1.52o, NIH), based on distance-to-pixel proportion. This data was analyzed with a MATLAB (MathWorks) algorithm to obtain cell numbers and statistical data for the sample. Nondisrupted cells were considered to have maintained the same roundness ratio and average surface after the disruption. The microalgal cell disruption rate ( $\tau_D$ ) refers to the complement proportion of the cells counted after disruption, compared to those counted before.

## 2.5 | Dry weight measurement

A 10 ml samples were filtered through previously-weighted glass fiber filters (0.45  $\mu\text{m}$ , Whatman GF/F, GE). The filters were then washed first in 1.19 M  $\text{NH}_4\text{HCO}_2$  and next in MilliQ water. The filters and the retained biomass were dried at 103°C for 1 h (minimum time required for achieving weight stabilization) and then weighed. The values reported



**FIGURE 1** Flat-panel airlift photobioreactors systems used for indoor cultivation. (a) 170 L HECtor system 170 L and (b) 1 L lab scale flat-panel airlift.

here as biomass concentration ( $X$ ) correspond to the mean values for a triplicate assay. These were calculated based on the weight difference between dry-biomass and clean filters per unit of culture volume used. The volumetric biomass productivity ( $P_X$ ) and areal productivity ( $S_X$ ) were also calculated, using the Pruvost, Cornet et al. (2011) method.

## 2.6 | Stress index

To compare the different physiological states, stress index was measured using pigment content quantification, as developed by Ritchie (2006) and Strickland and Parsons (1968). Stress index is an assessment of the carotenoids-to-chlorophyll ratio as an indicator of nitrogen depletion, and has also been found to correlate with the carbon-to-nitrogen ratio content of the cell independent of the nitrate in the medium (Heath et al., 1990).

First, 0.5 ml samples were collected and centrifuged at 6000 RFC, 4°C for 10 min. The supernatant was removed and replaced with 1.5 ml of methanol anhydrous 99.8%. The samples were then sonicated for 1 min and incubated at 45°C in the dark for 50 min. Immediately after this, the samples were again centrifuged and the light absorption of the supernatant was measured (V-730, Jasco). The stress index was then calculated as the ratio 480/662 nm minus the background absorption at 750 nm.

## 2.7 | Total fatty acids (TFA)/TAG content measurement and released TAG value

The TFA and TAG content of the cells were measured using the whole-cell method and analysis, published by Van Vooren et al. (2012). In brief, samples were centrifuged for 10 min at 3600 RFC and 4°C, then freeze-dried for posterior batch analysis at the end of each experiment run. The freeze-dried biomass was mixed with 6 ml of chloroform/methanol 2:1 v/v (Thermo Fisher Scientific) and 0.01% w/w of butylated hydroxytoluene, then incubated and agitated for 6 h at 25°C. The extract was recovered and analyzed for fatty acids by gas chromatography with a flame ionization detector (GC-FID, Thermo Fisher Scientific), and for TAG by high-performance thin-layer chromatography, (HPTLC, CAMAG). TFA and TAG measurements are expressed as a percentage of their mass to the processed algal biomass treated (TFA%<sub>X</sub> and TAG%<sub>X</sub>, respectively). The volumetric and areal productivities for TAG ( $P_{TAG}$ ,  $S_{TAG}$ ) were then calculated as described by Pruvost, Cornet et al. (2011). Additionally, the released TAG value was expressed as the product of TAG productivity (in kg/m<sup>3</sup>d or kg/m<sup>2</sup>d) and the cell disruption rate (i.e., Released TAG =  $P_{TAG} \cdot \tau_D$ ).

## 2.8 | Nitrate concentration measurement

The protocol proposed in the Standard Methods for the Examination of Water and Wastewater document (APHA, 2012) was used to

measure nitrate (NO<sub>3</sub><sup>-</sup>) concentration. This is based on the ultraviolet light absorbance of NO<sub>3</sub><sup>-</sup> at 220 nm using silica cells of 1 cm light-path. Organic matter may absorb ultraviolet light on the same wavelength. To prevent this, an additional measurement at 275 nm and preliminary filtration is required.

A sample of 1. ml of culture was diluted 20 times using highly-purified water and then centrifuged for 10 min at 3600 RFC and 4°C. The supernatant was then filtered using a 0.2 μm acetate cellulose filter (Minisart). The ultraviolet absorbance (V-730, Jasco) was measured immediately at the established wavelengths. Corrected sample absorbances were calculated using the equation  $ABS_{220nm} - 2 \times ABS_{275nm}$ . The values were then transposed into mM with a standard curve ( $r^2 = 0.999$ ) which used 1:20 diluted ASW depleted medium as a blank, and individual additions of NaNO<sub>3</sub> for the range 0–200 μM.

Values shown as  $N_t$  are for NO<sub>3</sub><sup>-</sup> concentration in the culture for a given sampling time.

## 2.9 | Experiment layout

The experiments carried out for this study were as follows:

- I. *Study of released TAG value in batch culture in DNC:*
  - (a) *Depleted culture in simulated DNC.* A 170 L indoor PBR was filled with depleted medium. This reactor was run in batch mode for 11 days and monitored for biomass concentration, cell disruption rate, and pigment concentration. Stationary growth phase was declared when dry weight measurements remained almost unvaried in time. The culture entered the stationary growth phase after the fifth day. This data was used to calculate productivity and released TAG values.
- II. *Study of the released TAG value in continuous N-limited culture and simulated DNC:*
  - The 1 L DNC-simulated indoor PBR was run in continuous mode under several dilution rates. The protocol for the addition of nitrate changed according to the following:
    - (b) *Minimum nitrate (NO<sub>3</sub><sup>-</sup>) concentration for sustained biomass growth and TAG accumulation.* For this experiment continuous PBR were arranged at different nitrogen concentration levels in the feeding medium ( $N_0$ ), each with multiple dilution rates  $D$ , as follows: for 56%NO<sub>3</sub> 0.04, 0.03, 0.02, 0.01 and 0.005 h<sup>-1</sup>; for 46%NO<sub>3</sub> 0.013, 0.01, and 0.007 h<sup>-1</sup>; for 29%NO<sub>3</sub>: 0.013, 0.01, 0.007, and 0.005 h<sup>-1</sup>. Biomass concentration and residual NO<sub>3</sub><sup>-</sup> were monitored for every set of experiments. Finally, biomass concentration, TFA/TAG, and pigment content were measured once the periodic regime was established. These values were used to calculate the daily nitrate consumption and TAG and biomass productivity.
    - (c) *TAG production with pulsed-addition of nitrate.* Once the dilution rate and daily nitrate consumption were established, it was decided to replace continuous nitrate addition with a pulsed-addition. ASW + CONWAY medium was used for this



and for continuous addition, but without the nitrate source. An individual solution of  $\text{NaNO}_3$  was prepared and added separately. The bulk medium was constantly supplied at 0.078 ml/min. Nitrate was supplied at the same ratio but for 34 min only, just before the start of a new daytime period (i.e., starting at 23:26 h the previous night). Inlet rates were adjusted to match the selected dilution rate, as in the section above ( $D = 0.005 \text{ h}^{-1}$ ). This rate was set based on the amount of nitrate needed to sustain daily cell growth and the limitations of the pumping device. Residual nitrogen, biomass concentration, cell disruption rate, and TAG content were monitored.

Note that the term *periodic regime* refers to the repeated changes in cell physiology that occur every DnC in continuous PBR culture mode. The periodic regime was declared once biomass concentration, nitrogen uptake, or TFA/TAG content remained unvaried for each sunset-sunrise cycle.

### 3 | RESULTS AND DISCUSSION

#### 3.1 | Released TAG in depleted batch culture and simulated DnC

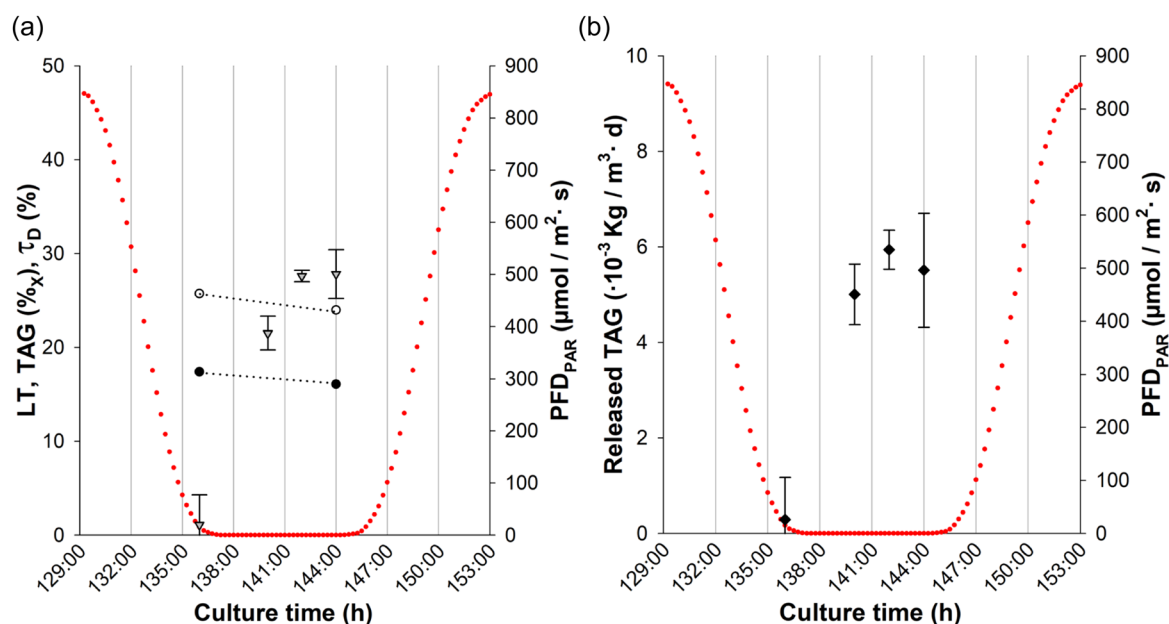
Previous results in outdoors conditions (data not shown) suggest that time-varying culture conditions in DnC could impact the mechanical resistance of the cells, and then the recovery of lipids in the subsequent downstream process. This indicates the existence of an

optimum harvesting point in DnC regimes. To limit the influence of pH and temperature, these were completely controlled in simulated DnC at laboratory scale to determine the release of TAG (Section 2.9, I-a).

The depleted batch culture in simulated DnC achieved stationary growth phase after 120 h. The final three night periods (112–168 h) were considered as stable cycles since biomass concentration remained between 0.9 and 0.8  $\text{kg}/\text{m}^3$  ( $\text{SE} = 0.04$ ,  $n = 3$ ) each day, and other measured quantities (TFA, TAG, and  $\tau_D$  values) also varied in a reproducible way. Because of the variation of TFA, TAG, and  $\tau_D$  during the night period (Figure 2a), the released TAG values were not constant either (Figure 2b). To note that Figure 2 only shows a sample of the TFA, TAG, and  $\tau_D$  values which varied in a reproducible way in the same interval for the final three night periods.

Figure 2a shows that TFA and TAG were consumed each night from 25 to 23% $\chi$  ( $\text{SE} = 0.7$ ,  $n = 3$ ) and from 17 to 16% $\chi$  ( $\text{SE} = 0.2$ ,  $n = 3$ ), respectively. These consumption values are in accordance with trends published by several works for different microalga species (Edmundson & Huesemann, 2015; Grobbelaar & Soeder, 1985; Lacour et al., 2012). TAG volumetric and surface productivities, for these consumption ranges, were therefore calculated in  $26\text{--}19 \times 10^{-3} \text{ kg}/\text{m}^3 \text{ d}$  ( $\text{SE} = 2.2$ ) and  $1.3\text{--}0.9 \times 10^{-3} \text{ kg}/\text{m}^2 \text{ d}$  ( $\text{SE} = 0.1$ ), respectively.

Meanwhile, the cell disruption rate  $\tau_D$  in simulated DnC was confirmed to vary. The cell disruption rate increased from 1.1% at the end of the day to 27.8% at the end of the night period (Figure 2a). These results corroborate those found by Angles et al. (2017) revealing higher resistance to disruption of microalgae in nitrogen depleted conditions with also large variations during DnC.



**FIGURE 2** Released TAG values in simulated day-night cycles in batch culture. The time range given represents measurements for 3 repetitive days during the periodic regime. (a) TFA, TAG content and  $\tau_D$ . The gray triangles represent  $\tau_D$ , the white circles TFA content and the black circles TAG content. (b) The released TAG value as black diamonds. Error bars for SE ( $n = 3$ ) (Section 2.9, I-a). TAG, triacylglycerol; TFA, Total fatty acid

Microalgae are not expected to maintain a homogeneous and stable physiological state during any DnC. Beacham et al. (2014) showed that some species of the *Nannochloropsis* genera experience an increase in cell wall thickness as a physiological response to stress conditions, which directly impacts lipid recovery. In the same manner, Scholz et al. (2014) suggested that algaenan synthesis is responsible for mechanical resistance in the cells in *N. gaditana*. All of these responses could be affected by light, temperature, and nitrate availability.

On this regard, monitoring the synthesis of algaenan in the cell wall would be an interesting perspective for future research. This may be achieved based on the study of algaenan precursors ( $C_{18}$  fatty acids) or elongation enzymes into  $C_{28}$ - $C_{34}$  (such as polyketide synthases) during nitrogen starvation and DnC (Gelin et al., 1997; Janssen et al., 2020; Janssen, Lamers, et al., 2019; Scholz et al., 2014; Zhang & Volkman, 2017).

The combined results for TAG consumption and cell disruption from the depleted batch culture in simulated DnC, enable to calculate the released TAG (Figure 2b). A maximum released TAG value of  $5.9 \times 10^{-3}$  kg/m<sup>3</sup>d (SE = 0.41,  $n = 3$ ) ( $0.3 \times 10^{-3}$  kg/m<sup>2</sup>d) was achieved at 270 min after the end of the day. In other words, TAG content is maximum as soon as the daytime period is over since the cells have not yet started the respiration process. However, not all the TAG can be recovered because the cells are more resistant to mechanical disruption at this point. By the end of the night, therefore, the cells are less resistant but have already catabolized some of the TAG. This indicates the presence of an optimum harvesting time, identified here to be after 4.5 h (270 min) when the TAG released was found to be at a maximum for the DnC. If the biomass is harvested and disrupted at the onset of night, around 90% of the TAG production may be lost due to the mechanical resistance of the cell being too great.

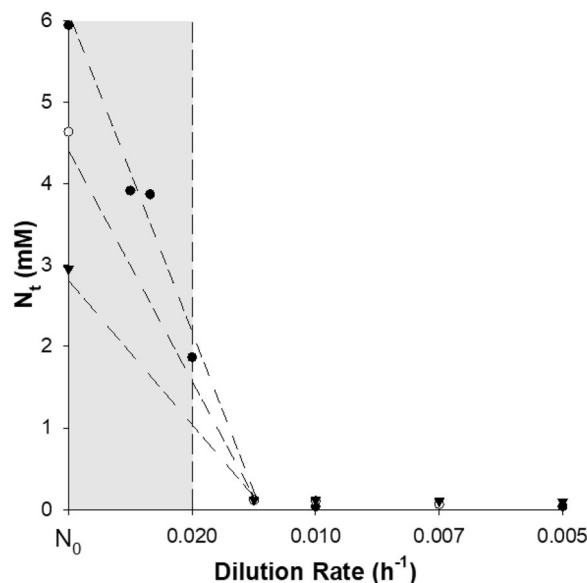
The concept of released TAG, presented by this study as a function of the variations in mechanical resistance during DnC, is therefore found to be a significant element for optimizing TAG production.

### 3.2 | Released TAG in continuous N-limited culture and simulated DnC

#### 3.2.1 | Minimal initial $NO_3^-$ concentration for TAG accumulation

The possibility of establishing a culture operated in continuous mode (for continuous production), under nitrogen limitation (for simultaneous TAG accumulation and cell division), and in simulated DnC (for outdoor cultivation) was then studied (Section 2.9, II-b,c).

First, to sustain cell growth while accumulating TAG in simulated DnC, it was established the minimum initial nitrate concentration  $N_0$  at the maximum dilution rate without washing out the system. Figure 3 shows the residual nitrogen  $N_t$  trend observed in the periodic regime with three different initial nitrogen concentrations (56% $NO_3$ , 46% $NO_3$ , and 29% $NO_3$ ) for six different dilution rates. When the dilution rate was higher



**FIGURE 3** Residual  $NO_3^-$  concentration for the periodic regimes at different dilution rates in simulated day–night cycles. Plots are for three different initial nitrate concentrations with continuous addition: 56% (5.94 mM) (black dots), 46% (4.63 mM) (white dots) and 29% (2.95 mM) (black triangles). The gray zone represents dilutions rates where culture was washed out ( $n = 1$ ) (Section 2.9, II-b)

than  $0.02 \text{ h}^{-1}$ , the cultures washed out even at higher nitrate concentrations. The other cultures, with dilution rates equal to or slower than  $0.013 \text{ h}^{-1}$ , arrived at the periodic regime with residual nitrogen  $N_t$  close to zero (about to  $0.03 \text{ mM}$  for 56% $NO_3$ ,  $0.11$ – $0.06 \text{ mM}$  for 46% $NO_3$ , and  $0.2$ – $0.10 \text{ mM}$  for 29% $NO_3$ ). These differences are mainly based on the combined effects of dilution rate and growth rate resulting from each nutrient limitation. When the dilution rate is high enough, biomass loss will prevail over cell growth (due to large biomass losses through harvesting), but when the dilution rate is lower than  $0.02 \text{ h}^{-1}$ , the growth rate compensates for biomass losses (due to both harvesting and night catabolism) and the culture is, therefore, able to reach a periodic regime.

However, the highest TAG productivities were found for cultures with an initial nitrogen concentration of 29% $NO_3$ . For this condition, results for the high ( $0.013 \text{ h}^{-1}$ ) and low ( $0.005 \text{ h}^{-1}$ ) dilution rates are presented in Table 1. These cultures reached the periodic regime after 16 days, which was maintained until the end of the experiment, 15 days later. At a dilution rate of  $0.013 \text{ h}^{-1}$ , TAG surface productivities of 2.9 and  $2.3 \times 10^{-3} \text{ kg/m}^2\text{d}$  at the sunset and sunrise sampling times were obtained respectively. Likewise, the system at a dilution rate of  $0.005 \text{ h}^{-1}$  enabled to obtain a slightly higher TAG surface productivity of 3.0 and  $2.5 \times 10^{-3} \text{ kg/m}^2\text{d}$  for the same sampling times.

Variations in productivity are consequences of the DnC and the stress caused by the different levels of nitrogen depletion in each system. As a result, cells grow during the daytime period and consume energy reserves during the night period (losing about 3%–4% $X$  of TAG). The stress indexes of present cultures (from sunset to sunrise) remained within the range 0.75–0.72 for the  $0.005 \text{ h}^{-1}$  condition, and from 0.68 to

**TABLE 1** Biomass concentration, TFA and TAG content, TAG surface productivity and stress index for two dilution rates obtained in the periodic regime of simulated day–night cycles

D (h <sup>-1</sup> )	Sampling time	X (kg/m <sup>3</sup> ) (SE)	TFA% <sub>x</sub> (SE)	TAG% <sub>x</sub> (SE)	S <sub>TAG</sub> (10 <sup>-3</sup> kg/m <sup>2</sup> d) (SE)	Stress index
0.005	Sunset	2.5 (0.04)	34 (2.3)	33 (3.0)	3.0 (0.78)	0.75
	Sunrise	2.3 (0.05)	34 (1.6)	30 (1.7)	2.5 (0.19)	0.72
0.013	Sunset	1.3 (0.04)	25 (1.4)	24 (1.0)	2.9 (0.21)	0.68
	Sunrise	1.2 (0.05)	23 (0.9)	20 (3.3)	2.3 (0.42)	0.61

Note: Initial NO<sub>3</sub><sup>-</sup> concentration was set at 29% (2.95 mM). (Section 2.9, II-c).

Abbreviations: TAG, triacylglycerol; TFA, Total fatty acid.

0.61 for the 0.013 h<sup>-1</sup> condition. These values emphasize that the present culture strategy enables an equilibrium of physiological states that allows cells to both divide and accumulate lipids in DNc and continuous mode. The condition at the dilution rate 0.005 h<sup>-1</sup> resulted in the highest productivity, producing 2.5 – 2.3 kg/m<sup>3</sup> of biomass concentration (biomass surface productivity of 9–8.2 × 10<sup>-3</sup> kg/m<sup>2</sup>d) with high TAG content of 33%–30%<sub>x</sub>, compared to the dilution rate 0.013 h<sup>-1</sup> which produced only 1.3–1.2 kg/m<sup>3</sup> of biomass concentration with a lower TAG content at 24–20%<sub>x</sub>.

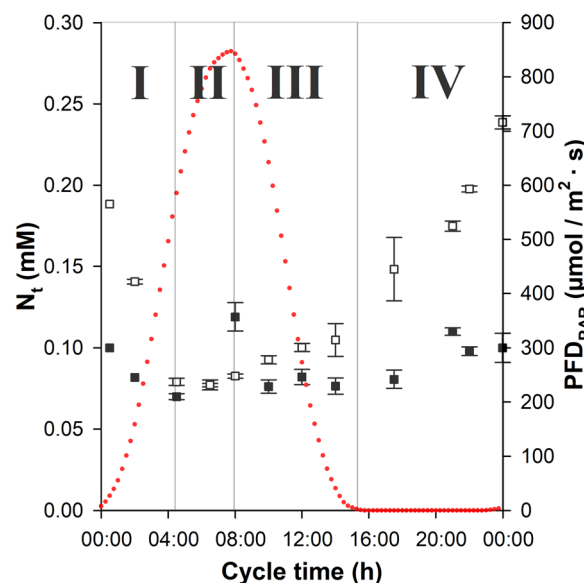
Differences in TAG content could also have been due to the time-lapse the cells experienced in nitrogen starvation. Figure 4 shows a sample of residual nitrogen N<sub>t</sub> in the culture medium during the DNc. For an initial 29%NO<sub>3</sub> concentration in the medium, a minimal residual N<sub>t</sub> value of around 0.07 mM was obtained during a 24 h cycle. This could be due to the existence of a basal nitrogen concentration allowing for cell growth, or the minimum detection range of the analytic method. Whatever the reason, four daily phases can be observed. These phases were found to be repeated and unvaried from the beginning of the periodic regime until the end of the culture (15 days):

Phase I. *Fast nitrogen consumption*. Microalgae start their metabolic activity with a maximum nitrogen concentration in the medium and increasing light source (onset of the day). As a result, nitrogen concentration decreases while biomass increases.

Phase II. *Late nitrogen consumption*. Residual nitrogen reaches a minimum value after 4 h of increasing light (midday). Cells may metabolize at or near their maximum rate to achieve nitrogen depletion.

Phase III. *Early nitrogen accumulation*. As a consequence of a presumable reduced photosynthetic activity after peak light (possible photo-saturation), metabolic activity is slower and the nitrogen concentration in the medium starts to accumulate again due to the constant supply; but the nitrogen source is still consumed (end of the day).

Phase IV. *Full nitrogen accumulation*. At this point, photosynthetic growth stops since there is no more light to activate the growth metabolism. Respiration begins and the nitrogen source starts to accumulate almost linearly in the medium due to constant feeding (night period).



**FIGURE 4** Residual NO<sub>3</sub><sup>-</sup> concentration in the periodic regime in simulated day–night cycles for two dilution rates. Plots are for dilution rates 0.005 h<sup>-1</sup> (black squares) and 0.013 h<sup>-1</sup> (white squares). Initial NO<sub>3</sub><sup>-</sup> concentration was set at 29% (2.95 mM). The red dots represent a simulated day–night cycle. The time range given represents measurements for 3 repetitive days during the periodic regime (error bars for SE, n = 3) (Section 2.9, II-b)

Likewise, Figure 4 shows the increase in nitrogen consumption that occurs during the light period. Throughout, all along the 24 h cycle, cultures were found to consume 0.33 mM (28.29 mg) and 0.84 mM (71.73 mg) of NaNO<sub>3</sub> at 0.005 and 0.013 h<sup>-1</sup>, respectively.

### 3.2.2 | TAG production under a pulsed-addition of NO<sub>3</sub><sup>-</sup>

Based on the observations of nitrate consumption phases, the previous operational parameters were modified for further optimization (Section 2.9, II-c). The objective was to apply a pulse addition of NO<sub>3</sub><sup>-</sup> to avoid its accumulation during the cycle, to obtain a longer starvation period during DNc than in the previous experiments. The amount of NO<sub>3</sub><sup>-</sup> known to be consumed in 24 h (corresponding to a



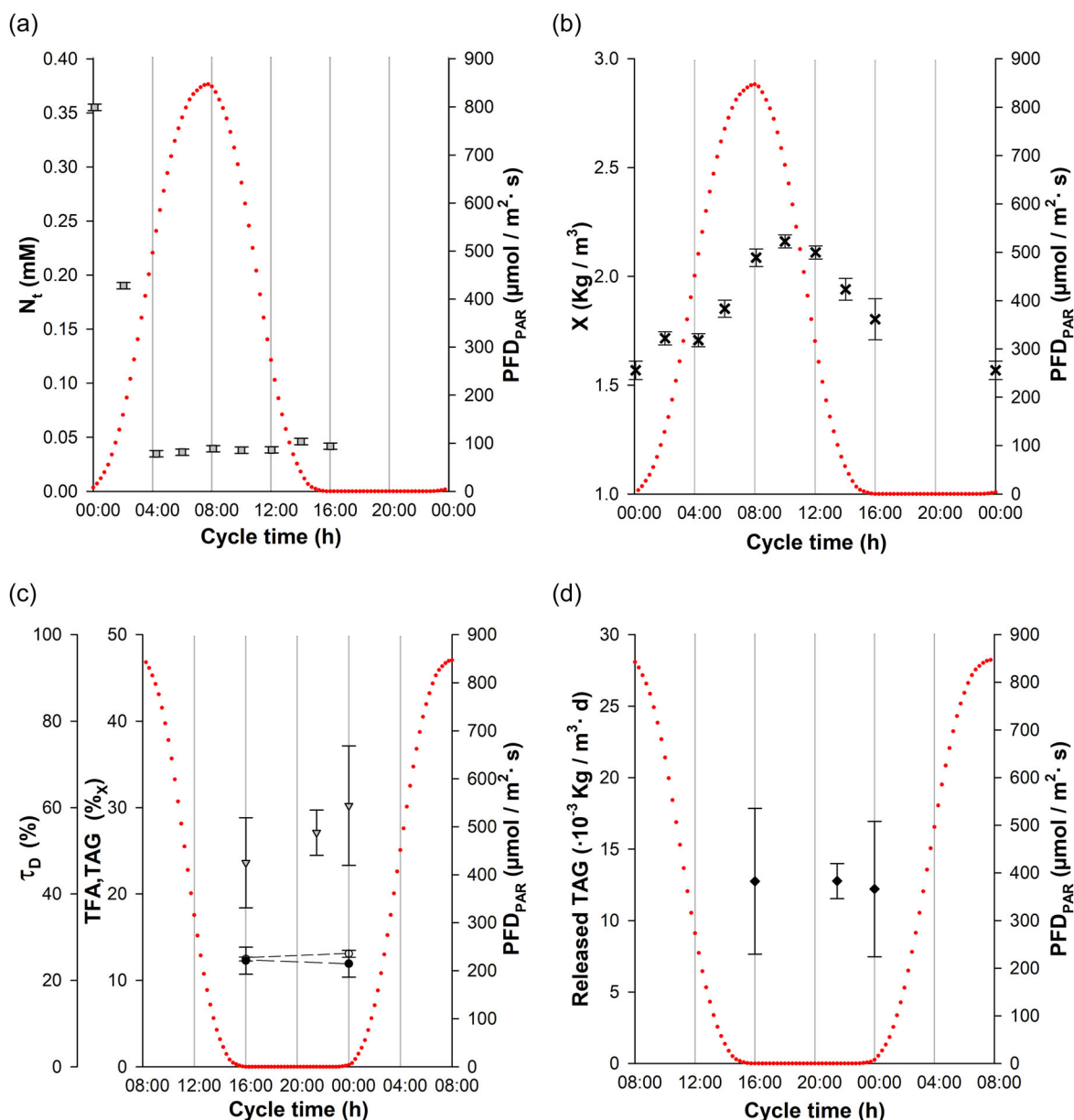
29% $\text{NO}_3^-$  limitation at  $0.005^{-1}$ ) was added separately from the bulk medium in a single pulse, only at the onset of each daytime period (i.e., at the beginning of phase I: fast nitrogen consumption). This  $\text{NaNO}_3$  supply protocol was applied once the reactor achieved the periodic regime.

Janssen et al. (2018) ran a similar protocol with *N. gaditana* batch culture. A pulsed-dose of  $\text{KNO}_3^-$  (0.23 M) was added at the beginning of each night period during the stationary growth phase. For the night period 498.5 and 674.3 mg of the solution was added to help the recovery of photosystems, which was compromised in nitrogen starvation due to lipid transposition. In contrast, the present study

added  $\text{NaNO}_3$  based on the nitrogen requirement previously determined, so that it was entirely consumed before sunset.

This culture protocol took 28 days to reach the periodic regime and continued unchanged for another 29 days, revealing a stable regime despite the stress conditions applied. As with previous experiments, once in this state, the culture measurements were done periodically until the end of the experiment. These repetitive cycles are discussed below.

Figure 5a shows the residual nitrogen  $N_t$  monitored for 24 h. As expected, it took 4 h for the microalgae culture to consume the supplied nitrogen. This is similar to the corresponding phase I (from 00:00 to 04:00 h) described in the previous section. Without the



**FIGURE 5** Culture changes for a pulsed-addition of  $\text{NO}_3^-$  in continuous mode in day-night cycles. The time range given represents measurements for 3 repetitive days during the periodic regime. In (a) gray squares represent residual nitrogen  $N_t$  (error bars for SE,  $n = 3$ ); in (b) the black crosses represent dry weight biomass measurement (error bars for SE,  $n = 3$ ); in (c) gray triangles represent the cell disruption rate  $\tau_D$  (error bars for SE,  $n = 25$ ) and the white and black circles represent TFA and TAG content respectively (error bars for SE,  $n = 3$ ); and in (d) the black diamonds represent the released TAG (error bars for SE,  $n = 23$ ) (Section 2.9, II-c)

**TABLE 2** Overview of the main experiments in simulated DNc

Culture system	TAG (%)	$\tau_D$ (%)	$S_X$ ( $10^{-3}$ kg/m <sup>2</sup> d)	$S_{TAG}$ ( $10^{-3}$ kg/m <sup>2</sup> d)	Released TAG ( $10^{-3}$ kg/m <sup>2</sup> d)
Depleted batch	17–16	1.1–27.8	7.64–5.9	1.3–0.9	0.3
Continuous nitrogen addition	33–30		9–8.2	3–2.5	1.4–1.5
Nitrogen pulsed-addition	12.3–11.9	47–60	6.6–5	0.8–0.6	0.4
Nitrogen pulsed-addition (half dose)	12.1		2.5	0.3	0.1–0.2

Note: Data was obtained from the periodic regime of the cultures, except for the depleted batch which was obtained from the stationary growth phase.

continuous nitrogen supply,  $N_f$  then remained at a minimal level of 0.04 mM.

Biomass growth is shown in Figure 5b. It seems to confirm previous phases observed under DNc. A lag-like phase can be distinguished (Phase I, from 00:00 to 04:00 h) where cells might repair their photosystems and use the nitrogen source consumed, presumably to synthesize new proteins (Janssen et al., 2018). An exponential phase, Phase II (04:00–10:00 h) can then be observed with the rapid increase of biomass concentration from 1.7 to 2.1 kg/m<sup>3</sup>. This separation of the growth phase from the nitrogen uptake phase may be caused by the use of nitrogen intracellular pools (Dortch, 1982), which are accumulated when nitrogen is available and used by the cells when there is still light available for photosynthesis but no nitrogen in the medium. Rafay et al. (2020) also showed a similar result with *N. gaditana* in a repeated batch culture at 395  $\mu\text{mol}/\text{m}^2\text{s}$ . Next, Phases III and IV (from 10:00 to 24:00 h) were observed, with the decrease in biomass concentration from 2.1 kg/m<sup>3</sup> to the lowest level of 1.4 kg/m<sup>3</sup>, as the combined result of the decrease in light and absence of nitrogen in the PBR.

Figure 5c also shows the cell disruption rate  $\tau_D$  and TFA and TAG cell content. TFA and TAG were revealed to not vary as much as in previous experiments during the night period, in contrast to the disruption rate which was found to be affected. The TFA values changed slightly during night period from 12.5 to 13.1%<sub>x</sub> (SE = 0.67% and 1.25%,  $n = 3$ ) and TAG changed from 12.3 to 11.9%<sub>x</sub> (SE = 1.57% and 1.55%,  $n = 3$ ). In this case, TAG represented about 90% of the TFA. In addition, the disruption rate continued to increase by 13%, rising from 47% to 60% during the night period. Nonetheless, standard errors of measurements (SE = 10%,  $n = 3$ ) mean that the variations are not certain, but it can still be argued that the algaean (as a possible explanation to changes in mechanical resistance) might be affected by the DNc or nitrogen depletion. The changes in long-chain hydrocarbons in the cell wall composition under DNc therefore would help to confirm this hypothesis.

The calculated TAG volumetric and surface productivities ranged from 27.0 to 20.2  $\times 10^{-3}$  and 0.8 to 0.6  $\times 10^{-3}$  kg/m<sup>2</sup>d, respectively. These results are very similar to those from the batch in simulated DNc. However, the present protocol led to more interesting cell disruption rate values. The corresponding released TAG in the experiment is shown in Figure 5d. Constant values of around 12.6  $\times 10^{-3}$  kg/m<sup>3</sup>d (SE = 3.7,  $n = 3$ ) (0.4  $\times 10^{-3}$  kg/m<sup>2</sup>d) are obtained, which is approximately double that achieved with the batch PBR in simulated DNc.

The former is higher not just because of the difference in TAG content, but also because the disruption rate is not affected as much as in normal batch depletion (Figure 2), and also because of the higher biomass concentration achieved in the continuous culture.

Finally, a last experiment was carried out where half the nitrogen dose was supplied under exactly the same conditions (data not shown). The aim was to see if lower nitrogen concentrations would impact TAG content under this protocol. Surprisingly, after stabilization (31 days), the TFA and TAG content did not alter significantly (12.4% and 12.1%, respectively; SE = 0.23% and 0.82%,  $n = 2$ ) but biomass concentration was reduced to 0.7 kg/m<sup>3</sup> (2.5 and 2.1 kg/m<sup>3</sup> for the continuous and full pulsed-dose protocols, respectively). With the nitrogen requirement halved, the surface TAG productivity was reduced to 0.3  $\times 10^{-3}$  kg/m<sup>2</sup>d, which is also about half of the surface TAG productivity with the full nitrogen dose.

The comparison of TAG productivities obtained under continuous nitrogen addition (3–2.5  $\times 10^{-3}$  kg/m<sup>2</sup>d), and pulsed-additions at full nitrogen dose (0.8–0.6  $\times 10^{-3}$  kg/m<sup>2</sup>d) and half dose (0.3  $\times 10^{-3}$  kg/m<sup>2</sup>d), enable to conclude that the highest productivity (and the highest TAG content) was achieved under continuous addition, with values 70% and 90% larger than with the pulsed-addition protocols at full and half doses respectively (Table 2). It can be hypothesized that a small amount of nitrogen in the medium, compared to a fully-starved culture is still needed for the synthesis of TAG during the daytime period. It is possible that some translocation or de novo synthesis pathways switch on and off to allow the cell to survive or starve during DNc. Therefore, the decrease in productivity with a pulsed-addition would be due to an imbalance of TAG synthesis, resulting from the lack of nitrogen when triggering N-deprivation during the daytime period. A residual concentration of nitrate could therefore have a positive effect on TAG productivity.

## 4 | CONCLUSIONS

This study investigated nitrogen-limited continuous culture protocol to exploit the night-time dynamics of *N. gaditana* in outdoor culture conditions. Particular attention was paid to the variations in the cell disruption rate, and the dynamics of biomass growth and loss leading to TAG consumption during DNc. Using the concept of released TAG, the optimal harvesting time of a batch N-depleted solar culture was

calculated at 4.5 h (270 min) after sunset, corresponding to a released TAG of  $5.9 \times 10^{-3}$  kg/m<sup>3</sup>d ( $0.3 \times 10^{-3}$  kg/m<sup>2</sup>d). In continuous mode, reducing the dilution rate ( $0.005 \text{ h}^{-1}$ ) with a limited nitrogen supply was found to successfully balance cell growth and TAG accumulation enabling to obtain a stable periodic regime, stable under simulated DnC. As a possible optimization of the protocol, a single dose of the nitrogen source applied at the onset of the light period was tested, but it was found to be less effective, as certainly explained by the need to maintain a minimum nitrogen concentration for a better synthesis of TAG. The best TAG productivity was in final achieved with continuous addition of an N-limited medium (around  $3 \times 10^{-3}$ ; and  $9 \times 10^{-3}$  kg/m<sup>2</sup>d of biomass surface productivity). Because of a larger cells disruption rate, the estimated released TAG of  $1.4 \times 10^{-3}$  kg/m<sup>2</sup>d was found 4.6 times larger when compared to batch culture. This emphasized the interest of such continuous protocol in the case of *N. gaditana* which exhibits a large decrease in cells disruption rate when submitted to nitrogen starvation, which could impair the overall production protocol efficiency.

## AUTHOR CONTRIBUTIONS

**Vladimir Heredia:** Conceptualization, Formal analysis, Investigation, Writing - Original Draft. **Luc Marchal:** Writing - Review & Editing. **Olivier Gonçalves:** Writing - Review & Editing. **Jeremy Pruvost:** Conceptualization, Writing - Review & Editing, Supervision.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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