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Repeated nitrogen starvation doesn't affect lipid productivity of *Chlorococcum littorale*

Iago Teles Dominguez Cabanelas [a,*], Dorinde M.M. Kleinegris [b], René H. Wijffels [a,c], Maria J. Barbosa [a]

[a] Wageningen University, Bioprocess Engineering, AlgaePARC, P.O. Box 8128, 6700 EV Wageningen, Netherlands
[b] Wageningen UR, Food & Biobased Research, AlgaePARC, Bornsesteeg 10, Building 112, 6721NG Bennekom, Netherlands
[c] Faculty Biosciences and Aquaculture, University of Nordland, N-8049 Bodø, Norway

HIGHLIGHTS

- *C. littorale* biomass productivity was stable under repeated N-starvation (147 days).
- *C. littorale* showed fast recovery of PSII after long repeated N-starvation.
- Repeated N-starvation cycles reduced biomass productivity, but not lipid content.
- Repeated short N-starvation cycles led to similar lipid productivities as batch N-starved cultures.

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ABSTRACT

In the present work we wanted to know what happens during time to biomass and lipid productivities of *Chlorococcum littorale* repeatedly subjected to N-starvation. Experiments were done using repeated cycles of batch-wise N run-out (after 2 days N = 0). Two different cycles were used: repeated short-starvation (6 days of N = 0) over a total period of 72 days and repeated long-starvation (13 days of N = 0) over a total period of 75 days. Batches (using fresh inocula) were done separately as control. Shorter and longer periods of starvation showed no differences in biomass productivities and PSII quantum yield evolution. The repeated short-starvation-batches showed the same lipid productivities as the control short-starvation batches. Most importantly, the biomass lipid content was the same between control and repeated-batches. Altogether, the results point to *C. littorale* as a resilient and stable strain, with potential to be used under semi continuous cultivation.

1. Introduction

Microalgae-based technology has the potential to supply new sustainable products due to its versatility (biomass can be simultaneously refined into multiple products) and sustainability (e.g., CO_2 neutral and no arable land required) (Ali Bahadar, 2013; Lam and Lee, 2012; Rawat et al., 2013). Microalgae products have various applications ranging from commodities (e.g., oils and carbohydrates for chemicals and fuels) to fine chemicals (e.g., rare fatty-acids and pigments) (Chisti, 2007; Norsker et al., 2011). Although microalgae products are a reality in the high-value market of fine chemicals, the current productivities and consequent high production costs do not allow them to be at present, a competitive feedstock for the low-market value of biofuels, for example (Pienkos and Darzins, 2009; Ter Veld, 2012; Wijffels and Barbosa, 2010). Microalgal lipids are one of the most versatile among microalgal products with uses ranging from commodities to food supplements and animal feed (Jobling and Bendiksen, 2003; Klok et al., 2014).

There are two main approaches to increase microalgal lipid volumetric productivity: process optimization and strain improvement. Process optimization is used to maximize the production of a certain compound using already available strains (Saha et al., 2013). Strain improvement is used to select cells that display an increased baseline production of a certain compound, which can be achieved via breeding and artificial selection (Combe et al., 2015; Fu et al., 2013; Portnoy et al., 2011) and/or mutagenesis (de Jaeger et al., 2014; Doan and Obbard, 2012; Velmurugan et al., 2014). A combination of process optimization and strain improvement could significantly increase productivity and reduce production costs.

Batch cultivation is the most commonly used strategy for microalgal lipid production (Feng et al., 2011; Klok et al., 2014;

* Corresponding author.
E-mail address: iago.dominguezteles@wur.nl (I.T.D. Cabanelas).
URL: http://www.AlgaePARC.com (D.M.M. Kleinegris).

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Münkel et al., 2013; Zemke et al., 2010). The traditional approach for microalgae lipid production works in 2 steps: microalgae biomass is produced in one system (growth phase) and then transferred to a second system where lipid production is induced by nitrogen starvation ("stress" phase), usually via dilution in N-free medium (Griffiths et al., 2012; Velmurugan et al., 2014). Instead of always producing a new inoculum to start a new batch for lipid production by N-starvation, it has been suggested that a repeated batch process using part of the produced lipid-rich biomass as inoculum could yield higher lipid productivities (Wang et al., 2015). This would remove the need for systems allocated for biomass production (growth phase), which consume production time and area that otherwise could be allocated to actual lipid production processes. However, the repeated batch approach could also lead to reduced productivity, due to a higher risk of operational crashes and contaminations. Furthermore, exposure to identical conditions can also lead to changes/mutations in the original strain, a feature that can be exploited to develop new strains with improved phenotypes (Portnoy et al., 2011).

Interestingly, repeated batch can also be used in laboratory evolution experiments, in which a cell population is subjected to a repeated environmental pressure to push for changes leading to a new, improved strain (Portnoy et al., 2011). Although such approach has been extensively used with *E. coli* and *S. cerevisiae*, only recent results have shown the potential with microalgae (Fu et al., 2013, 2012; Yu et al., 2013).

In the present work we wanted to know what happens during time to biomass and lipid productivities of *Chlorococcum littorale* repeatedly subjected to N-starvation. *Chlorococcum littorale* was selected for the current work based on its high photosynthetic activity under N-starvation and high TAG content (Benvenuti et al., 2015; Hu et al., 1998). Current microalgal lipid production relies on cycles of N-starvation repeated batches, which makes the above question relevant for lipid production by microalgae. Our work focusses on comparing two process – batch (reference strategy) and repeated-batch – to evaluate two aspects of the same question: 1] are the biomass and lipid productivities in the repeated-batch different from the control batch? and 2] does repeated-batch result in stable biomass and lipid productivities through time? We discuss, at last, the implications of our results to ALE experiments.

2. Materials and methods

2.1. Microorganism and culture conditions

Chlorococcum littorale (NBRC 102761) was purchased from the culture collection of NIES, Japan. This strains has been described by (Chihara et al., 1994; Ota et al., 2009). Artificial seawater medium was used to prepare inoculum and to perform experiments in reactors. Medium composition (g l^{-1}): NaCl 24.55; $MgSO_4{\cdot}7H_2O$ 6.60; $MgCl_2{\cdot}6H_2O$ 5.60; $CaCl_2{\cdot}2H_2O$ 1.50; $NaNO_3$ 1.70; HEPES 11.92; $NaHCO_3$ 0.84; EDTA-Fe (III) 4.28; K_2HPO_4 0.13; KH_2PO_4 0.04; trace elements in mg l^{-1}: $Na_2EDTA{\cdot}2H_2O$ 0.19; $ZnSO_4{\cdot}7H_2O$ 0.022; $CoCl{\cdot}6H_2O$ 0.01; $MnCl_2{\cdot}2H_2O$ 0.148; $Na_2MoO_4{\cdot}2H_2O$ 0.06; $CuSO_4{\cdot}5H_2O$ 0.01). The inoculum was grown in 0.2 L borosilicate Erlenmeyer flasks under controlled conditions (orbital-shaker-incubator, INFORS HT, Switzerland): mixing at 120 rpm, light intensity 150 μmol m^{-2} s^{-1}, temperature at 25 °C and CO_2 supply of 2% over air flow in the headspace.

N-starvation experiments were carried out in flat panel reactors as described by Breuer et al. (2013). The working volume of the reactors was 380 ml with a light path of 14 mm. Operation was performed at continuous illumination (incident light 410 μmol m^{-2} s^{-1}) using LED lamps with a warm white light spectrum (Bridgelux, BXRA W1200). The pH was kept constant at 7.0 by pulse-wise CO_2 addition to the airflow.

Nitrogen starvation was achieved via nitrogen run-out, i.e., cells were allowed to consume all available nitrogen (which happened after 48 h in the current experiments). The nitrogen content in the medium (N-NO_3 mg l^{-1}) was measured daily using the Sulphanilamide N-1-naphthyl method (APHA 4500-NO3-F) with an automatic analyser (SEAL AQ2).

2.2. Repeated N-starvation experiments

The repeated N-starvation experiments consisted of repeated cycles of batch-wise N run-out, i.e., at the end of each batch the reactor was diluted to the initial biomass concentration (OD750 of 0.5) with fresh, sterile nitrogen replete medium. The experiments followed the scheme presented in Fig. 1. Two different cycle durations were used, short cycle and long cycles, and for both cycles the first 2 days of cultivation where with nitrogen present (growth phase). After the first 2 days we considered the N-starvation phase, in which all nitrogen was taken up by the cells (arrow, Fig. 2). More information about the cycles: 8 cycles of repeated short-starvation (RSS, 6 days of N-starvation; grey points in Fig. 1 and 64 days in total) followed by 5 cycles of repeated long-starvation (RLS, 12 days of N-starvation; dark points in Fig. 1 and 70 days in total). The first 8 cycles were subsequent cycles of short-starvation and from the ninth cycle onward it was decided to increase the cycle duration to long-starvation to evaluate the effect of a longer period of starvation on *C. littorale*.

The cultivations from repeated N-starvation were compared with control experiments: 3 independent batches always starting with fresh inoculum made from plate to flask, all grown under the same conditions as previously stated. Each experiment was used to be compared with both Short-starvation and Long-starvation experiments. Hence, samples were taken on the 9th day of cultivation of each cycle to be compared with control short-starvation (CSS) and samples were also taken on the 15th day of cultivation to be compared with Long starvation-experiment (CLS).

Biomass concentration in the reactors was measured with optical density (OD) and gravimetric dry weight (DW). The OD measurements were a proxy of chlorophyll (680 nm) and biomass turbidity (750 nm), as stated by Griffiths et al. (2011). Samples were diluted within the range of the detection limit of the spectrophotometer (0.1–1 units). The dry weight (DW) measurements were done filtering 1–3 ml (triplicate) of microalgae culture on a

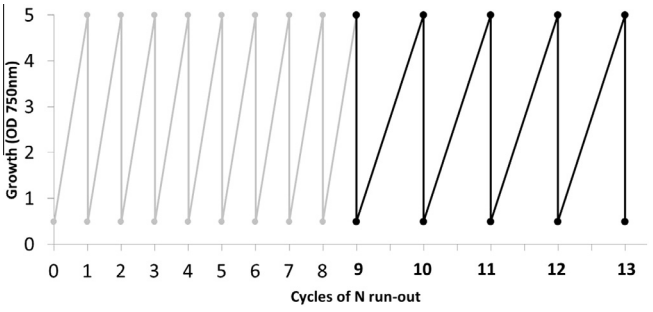

Fig. 1. Schematic representation of repeated N-starvation experiments. Each cycle represents one batch-wise N run-out cultivation composed of 2 phases: a growth or recovery phase (day 0 to day 2) and a N-starvation phase (from the day 2 onward). At the end of a cycle, biomass concentration was diluted to 0.5 (OD 750) and used as inoculum for the subsequent run. The grey points represent the repeated short-starvation period (RSS, 6 days of N-starvation, 8 cultivation days) and the dark points represent the repeated long-starvation period (12 days of N-starvation, 14 cultivation days).

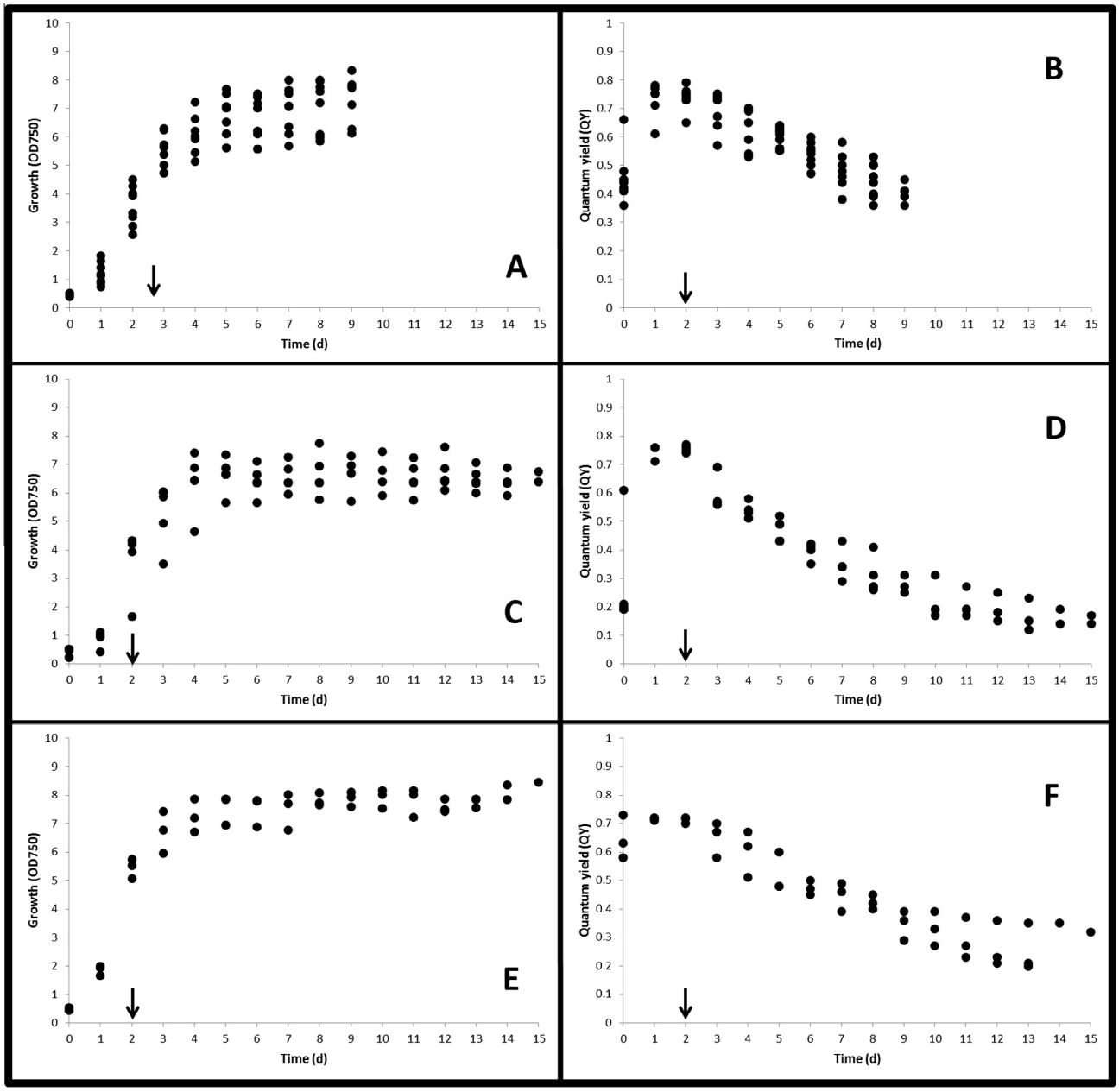

Fig. 2. Biomass production over time for repeated short-starvation (RSS, A), repeated long-starvation (RLS, C) and the control long-starvation (CLS, E). Time-evolution of the quantum yield of photosystem II (QY) for repeated short-starvation (RSS, B), repeated long-starvation (RLS, D) and the control long-starvation (CLS, F). The control short-starvation (CSS) is considered from day 0 to day 9 of Figures E and F. Arrows mark the beginning of the N-starvation period, which was the same for all experiments. Figures A and B represent the overlapped curves of 8 independent experiments (total 72 days), figures C and D represent 5 overlapped experiments (total 75 days) and figures E and F of 3 experiments (total 45 days).

previously dried (24 h, 100 °C) and weighted filter (GF/F Whatman®). After filtration the filters were set again to dry (24 h, 100 °C), and were finally weighted on a precision scale.

The quantum yield of photosystem II (QY) refers to the ratio between photons emitted and photons absorbed by the cells. The samples were kept between 0.1 and 0.8 OD units (750 nm), to keep cellular concentration within the machine range. Daily measurements were taken with a fluorometer (AquaPen-C AP-C 100, Photon System Instruments, Czech Republic). Samples were acclimated under a dark period of 10 min at room temperature. The F_v/F_m ratio gives the maximum QY of PSII (Eq. (1), from Benvenuti et al., 2015), where F_0 refers to the minimal level of fluorescence (after dark-acclimation) and F_m is the maximum fluorescence after exposing the cells to a pulse of actinic light.

$$(F_V/F_m) = \frac{F_m - F_0}{F_m} \quad (1)$$

2.3. Fatty-acids (FA) and triglycerides (TAG) analyses

Samples of biomass were harvested at the above mentioned time points. Biomass samples were centrifuged at 2500*g* for 5 min, washed twice with MilliQ® water and stored at −18 °C (supernatant was discarded). Subsequently, biomass samples were freeze-dried for 24 h. Triacylglycerides (TAG) and total fatty-acid (FA) extraction and quantification were done with the capillary column gas chromatographic method, as described by Breuer et al. (2013). Chemicals were acquired from Sigma Aldrich and

C15:0 (pentadecanoic acid, also from Sigma Aldrich) was used as internal standard for quantification.

2.4. Calculations

Biomass productivity (P_x, g L^{-1} d^{-1}) was calculated using Eq. (2),

$$Px = \frac{(Cx_{t_2} - Cx_{t_0})}{(t_2 - t_0)} \quad (2)$$

where the biomass concentration (C_x, g L^{-1}) of the reactor at the beginning (t_0, in days) and at the end of the growth phase (t_2, in days) were used.

Growth rate (μ) was calculated using Eq. (2),

$$\mu = \frac{Ln(Cx_{t_2} - Cx_{t_0})}{(t_2 - t_0)} \quad (3)$$

where the natural logarithmic (Ln) of biomass concentration (C_x, g L^{-1}) of the reactor at the beginning (t_0) and at the end of the growth phase (t_2) were used.

The decrease rate of the photosystem II (PSII) quantum yield (QY_r) was calculated using Eq. (4),

$$QY_r = \frac{Ln(QY_{t_f} - QY_{t_3})}{(t_f - t_3)} \quad (4)$$

where the natural logarithmic (Ln) of PSII quantum yield at two different points were used: the final day of starvation (t_f, which was t_9 and t_{15} for short and long starvation periods, respectively) and the beginning of the nitrogen run-out (t_2).

Total fatty-acids productivity (P_{FA}, g L^{-1} d^{-1}) was calculated using Eq. (5),

$$P_{FA} = \frac{(C_{FAtf} * C_{xtf})}{(t_f - t_3)} \quad (5)$$

where C_{FA} is the fatty-acid content in biomass (g g^{-1}) at the end of the starvation phase (t_f, which was t_9 and t_{15} for short and long starvation periods, respectively) and C_x is the biomass concentration (g L^{-1}) also at the end of the starvation phase. The total time of starvation (t, in days) is represented by the interval between the end (t_f) and the beginning (t_2) of the starvation phase.

Triacylglycerides productivity (P_{TAG}, g L^{-1} d^{-1}) was calculated using Eq. (5), with the difference that the fatty-acid biomass content (C_{FA}) was replaced by the triacylglycerides biomass content (C_{TAG}, g g^{-1}). Both FA and TAG were analysed to evaluate the effect of the starvation on the productivity of both reserve lipids (TAG) and polar lipids (considered as the difference between FA and TAG).

2.5. Data analysis

Null hypothesis significance testing (NHST) was used to compare the results between and among samples. On-way analysis of variance (ANOVA) was used to assess statistical significance within groups. Subsequently, to compare groups between and among each other, the one-step multiple comparison test of Tukey was used as a post-hoc test. In all cases the assumptions of the statistical testes were checked: normality was assessed with the Kolmorogov-Smirnov test and homoscedasticity among tested groups was assessed with Levene's Test. For both assumptions and hypothesis, the probability of type I error (α) was set at 0.05. All analyses were carried out with the software SPSS v.22.

3. Results and discussion

3.1. Biomass and lipid productivity under repeated N-starvation

The repeated N-starvation experiment was a continuous series of repeated batches that was divided into two phases: 8 cycles of short-starvation (RSS, 7 days of starvation) and 5 cycles of long-starvation (RLS, 13 days of starvation) (Fig. 1). The results obtained for each period were averaged and are presented in Fig. 2 and Table 1. No statistical differences were observed when comparing the growth rates (μ) and biomass productivities of RSS and RLS between and among each other (Table 1). This result shows two things: 1] the constant biomass productivity throughout repeated batches and that 2] doubling the starvation time showed no effect on subsequent biomass productivity in the next cycle.

The average growth rate from the repeated starvation periods (RSS and RLS) were also compared with the average growth rates of the controls (three independent batches). There was no difference in growth rate (μ) among all experiments (between 0.56 and 0.72 d^{-1}, Table 1). Similar growth rates show that *C. littorale* has a good capability of producing new functional biomass after N resupply, and was not affected by the repeated cycles of N-starvation. Comparable results were obtained with *Chlorella pyrenoidosa* using different intervals of harvesting followed by nitrogen resupply (Han et al., 2013).

Biomass productivity (Px), however, was statistically higher in the control batch experiments when compared with the repeated starvation cycles (1.52 g L^{-1} against 1.1 [RSS] and 1.0 g L^{-1} [RLS], Table 1). This result can be explained from the repeated N-starvation, since N is essential to produce new functional biomass (Adams et al., 2013; Mandalam and Palsson, 1998; Velmurugan et al., 2014). In repeated batches the biomass used to start a new cultivation has a lower nitrogen content in comparison with the biomass used to start a culture in the control batch. Thus, under the same conditions and equal nitrogen concentrations in the fresh medium, an inoculum with lower intracellular nitrogen content will reach lower values of biomass in comparison with the traditional batch, while retaining the same growth rate (Adams et al., 2013; Klok et al., 2013).

Other researchers have evaluated the effect of repeated-batch (with N run-out) on the biomass productivities of other species. *Chromochloris zofingiensis* showed a reduction from 0.75 to 0.66 g L^{-1} d^{-1} in biomass productivity when comparing the repeated-batch with the control batch, respectively (Mulders et al., 2014). Another related work (Benvenuti et al., 2015) evaluated the effect of different cycles duration on *Nannochloropsis* sp. under repeated batch in a lab-scale flat panel photobioreactor. In the work of Benvenuti et al. (2015), short repeated batches (1 day), showed an increase in biomass productivity from 0.68 (reference batch) to 0.94 g L^{-1} d^{-1}, while longer repeated batches (2 and 3 days), showed a reduction from 0.68 to 0.57 and 0.43 g L^{-1} - d^{-1}, respectively. Comparing results from different research is limited due to intrinsic variables (used species and experimental design). The analyses of our results and the above mentioned data, however, indicate that the nitrogen concentration and the duration of the starvation cycle play a major role when aiming at repeated-batch microalgae cultivation.

Additional to the growth rates and biomass productivities, the QY of PSII was also followed daily during the repeated cycles and control (Table 1; Fig. 2). The QYr was used to estimate the reduction rate in the PSII activity during N-starvation (Benvenuti et al., 2015; Griffiths et al., 2012). No statistical difference was found between batches with the same starvation period, but batches with longer starvation periods showed higher QYr (both, RSS and CSS; and RLS and CLS, Table 1). Our results show that the QYr is

Table 1
Growth parameters and productivities of *Chlorococcum littorale* under repeated N-starvation and control N-starvation experiments. For both cases both short and long starvation periods are presented. Different letters mean statistical significance (ANOVA, $p < 0.05$). Growth rate and biomass productivities were calculated between day 0 and 2. All other parameters were calculated using the N-starvation period (day 2 to the end of the cultivation). All productivities are presented in mg L^{-1} d^{-1} while the rates are per day (d^{-1}).

		Repeated N-starvation		Control N-starvation	
		Short (RSS)	Long (RLS)	Short (CSS)	Long (CLS)
Parameters					
Growth rate	μ	0.56 ± 0.12^{a}	0.60 ± 0.14^{a}	0.72 ± 0.12^{a}	0.72 ± 0.12^{a}
Biomass productivity	P_X	1.10 ± 0.26^{a}	1.02 ± 0.16^{a}	1.52 ± 0.19^{b}	1.52 ± 0.19^{b}
FA productivity	P_{FA}	0.28 ± 0.02^{a}	0.15 ± 0.02^{b}	0.33 ± 0.03^{a}	0.22 ± 0.01^{c}
TAG productivity	P_{TAG}	0.21 ± 0.03^{a}	0.12 ± 0.02^{b}	0.24 ± 0.05^{a}	0.18 ± 0.01^{a}
QY decrease rate	Qy_r	-0.21 ± 0.07^{a}	-0.07 ± 0.01^{b}	-0.19 ± 0.01^{a}	-0.09 ± 0.01^{b}
Growth phase	d	2	2	2	2
N-starvation phase	d	7	13	7	13
Total Cultivation time per cycle	d	9	15	9	15
Number of cycles		8	5	3	3

dependent on the duration of N-starvation and was not affected by the repeated starvation cycles. The QY values at the end of the stress period Fig. 2) were approximately 0.4 for the RSS and approximately 0.2 for the LRS, but those values returned to the same values of non-starved biomass (between 0.7 and 0.8) within 24 h of nitrogen re-supply. This finding indicates a quick recovery of photosynthetic capacity after N-resupply for *C. littorale*.

As it can be observed in Fig. 3, no statistical differences (ANOVA, $p > 0.05$) can be observed in the FA and TAG biomass content when comparing all four cycles among each other (the complete composition of both total fatty-acids and TAG's can be found in the Supplementary materials). As it can be seen, most lipids of *C. littorale* are synthesized within the first 6 days of starvation. After doubling the starvation period only 5% more lipids are synthesized (Fig. 3 and Table 1). Additionally to a stable lipid content, the saturation degree of both FA and TAG showed no differences when comparing all four cycles among each other starvation (Fig. 3A). In line with these previous findings, the biomass lipid content (both FA and TAG) shows that repeated N-starvation cycles over a period of 147 days did not affect the lipid metabolism of *Chlorococcum littorale*.

Lipid productivities (both FA and TAG) were stable throughout the repeated-starvation experiments with the same duration (both short and long starvation periods). The highest lipid productivities, however, were obtained for the short periods of starvation (ANOVA $p < 0.05$, Table 1). This result is explained by the response of microalgae when exposed to N-starvation: an increase in biomass lipid content is observed within the first hours/days after starvation starts (Griffiths et al., 2012). Lipid productivity (both FA and TAG) was the same between short repeated-starvation and short control starvation (RSS: 0.28 and CSS: 0.33 g L^{-1} d^{-1}, ANOVA $p > 0.05$ Table 1). Different results were obtained when comparing the experiments with long starvation periods: in this case the RLS showed statistically lower values of lipid productivity when compared with the CLS (0.15 against 0.22 g L^{-1} d^{-1}, respectively). Since lipid content was the same in both RLS and CLS, the smaller lipid productivity of RLS is explained by the lower biomass productivities obtained (a reduction from 1.5 [CLS] to 1.0 g [RLS] L^{-1} d^{-1}). These results show that the repeated N-starvation didn't impair lipid production and that for shorter periods of starvation *Chlorococcum littorale* showed the same lipid productivities as batch cultivations.

Altogether, our results show that repeated-batch is a suitable approach to produce *Chlorococcum littorale* for two reasons: 1] Under repeated N-starvation (short starvation cycles) TAG productivity was the same as the control-batch; and 2] Under repeated N-starvation (short and long cycles) both biomass and lipid productivities were stable throughout time.

3.2. Implications for adaptive laboratory evolution (ALE) experiments

Repeated batch operation is also used in experiments that aim at inducing changes in a population of microorganisms by repeatedly exposing them to stress factors (e.g. nutrient deprivation, competition for resources and abiotic factors like light and temperature). This approach is known as adaptive laboratory evolution (ALE), and the principle is that continuously diluting a microorganism culture under specific stress factors will cause random selection of cells (Portnoy et al., 2011). The expected goal is to have final populations that are more fit to the selective environment than the wildtype starting population. Previous work (Fu et al., 2012) showed an increase of 30% in biomass productivity of *Chlorella vulgaris* after 15 cycles (thus 45 days) of repeated batch (always with N) under red-LED lamps (6604 nm instead of

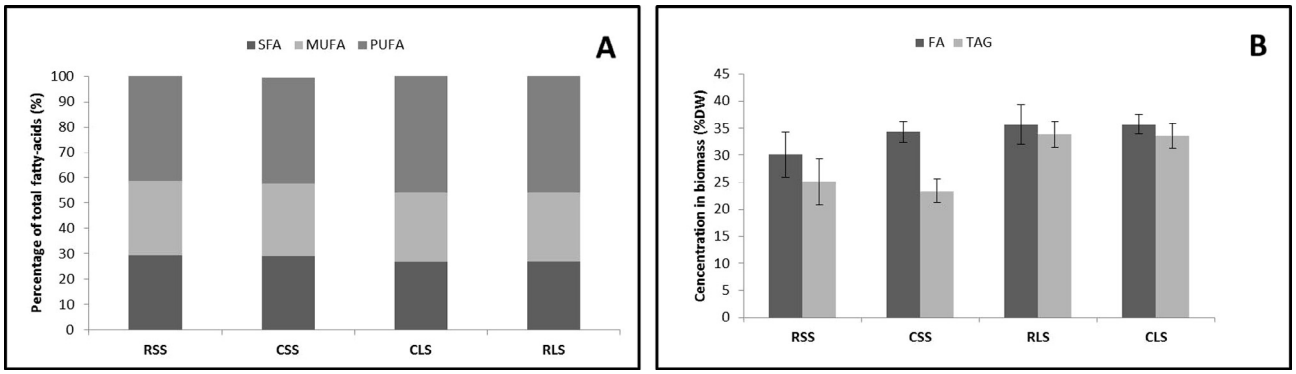

Fig. 3. Saturation degree of all 4 sets of experiments (A), the distribution shows the percentage (of total fatty acids) as saturated (SFA), monounsaturated (MUFA) en polyunsaturated (PUFA). (B) shows the final accumulated fatty-acids and TAGs for all 4 sets of experiments (shown as % dry weight). Acronyms are: RSS: repeated short-starvation; CSS: control short starvation; RLS: repeated long-starvation; CLS: control long starvation.

680 nm). Similarly, increased yield of β-carotenes was achieved with *Dunaliella salina* combining red and blue LED lamps under ALE experiments after 16 cycles (80 days) of repeated-batch (with N) (Fu et al., 2013). An ALE approach using CO_2 concentrations between 2% and 30% generated cell lines of *Chlorella sp.* with improved growth rate and chlorophyll contents after 31 cycles (97 days) under a CO_2 concentration at 10% (Li et al., 2015). Finally, improved growth (17–48% in comparison with Wt) was found in starch-less mutants of *Chlamydomonas reinhardtii* after 28 cycles of 3-day growth under constant light (Yu et al., 2013). Altogether, these previous works highlight the potential of some microalgae strains to be modified using adaptive laboratory evolution in a relatively short cycle of experiments, comparable to the duration of our experiments.

The possibility of improving microalgae performance in our experimental set-up was a secondary aspect of our research question. Although not a primary expectation from our work, the review above mentioned highlights the possibility to achieve cell lines of microalgae after relatively short periods of evolution (approximately 90 days). Another question is to access how stable are these differences shown by other authors, i.e. are these differences due to physiological adaptations or permanent mutations? Such question was not addressed the reviewed works above mentioned. The review of ALE experiments show that the most common response of microalgae to repeated stress is an increase in growth rate, which would increase the lipid productivity if the lipid metabolism is not affected (Fu et al., 2012; Li et al., 2015; Portnoy et al., 2011; Yu et al., 2013). In addition, the response to repeated stress (light, nutrients and temperature) is highly strain-dependent (Portnoy et al., 2011; Yu et al., 2013). With that in mind, one should pay attention to which abiotic/biotic variables can most potentially affect one's working strain.

Chlorococcum littorale showed a sharp and stable recovery in photosynthetic activity (QY) and nitrogen uptake rate immediately after N-resupply. At the same time the growth rates were maintained and lipid productivity of shortly starved biomass when compared with the control batch. Combined, these factors indicate a strain that can handle nitrogen environmental stress very well. In our experiments light was kept constant (400 μmols m^2 s^{-1}, in a reactor with 1.4 cm depth), while other reports have shown positive growth under 2000 μmols m^2 s^{-1} (1 cm flat panel reactor, 18 g L^{-1} cell density) (Hu et al., 1998). Since our experiments were aimed at studying the effect of nitrogen starvation, the light used was kept constant at 400 μmols m^2 s^{-1}. If aiming at inducing improved growth in *C. littorale* performance, one could consider ALE experiments under high light conditions or under sharp changes in light intensity, to introduce intensive selective pressure. Another option would be to use temperature as a selection pressure, since in the current experiments temperature was kept constant (at 25 °C) throughout the experiments. Previous work shown that *C. littorale* can grow from 16 to 32 °C, with optimum around 28 (Ota et al., 2009), showing that temperature might be a stress factor to induce the development of thermotolerant cell lines.

The stable performance of *C. littorale* shown in this work, indicates that this strain has a natural capability to handle fluctuations in the nutrient supply regime, which could indicate a possible strain to be used under fluctuating climate conditions. The resilience of *C. littorale* to repeated nitrogen starvation makes it suitable for repeated-batch cultivations and potentially unsuitable for inducing changes via ALE using N-starvation as a stress factor.

4. Conclusions

Shorter and longer periods of N-starvation didn't affect PSII quantum yield evolution and biomass productivity of *C. littorale*. Repeated short-starvation batches could be used as an operational strategy since it didn't affect lipid productivity. Longer periods of starvation, however, had lower lipid productivities when compared with the equivalent control. Most importantly, the biomass lipid content was the same between control and repeated batches, highlighting that the lipid metabolism was not impaired. Altogether, *C. littorale* is a resilient and stable strain, that can be cultivated in a semi continuous mode for both biomass and lipid production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.08.009.

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