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The effects of CO2-induced acidification on *Tetraselmis* biomass production, photophysiology and antioxidant activity: a comparison using batch and continuous culture

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Abstract :

A Tetraselmis sp. was selected for its antioxidant activity owing to its high lipid peroxidation inhibition capacity. With the aim to monitor culture conditions to improve antioxidant activity, effects of CO2-induced acidification on Tetraselmis growth, elemental composition, photosynthetic parameters and antioxidant activity were determined. Two pH values were tested (6.5 and 8.5) in batch and continuous cultures in photobioreactors. Acidification enhanced cell growth under both culture methods. However, the microalgae physiological state was healthier at pH 8.5 than at pH 6.5. Indeed, photosynthetic parameters measured with pulse amplitude modulated (PAM) fluorometry showed a decrease in the photosystem II (PSII) efficiency at pH 6.5 in batch culture. Yet, with the exception of the PSII recovering capacity, photosynthetic parameters were similar in continuous culture at both pH. These results suggest that lowering pH through CO2-induced acidification may induce a lower conversion of light to chemical energy especially when coupled with N-limitation and/or under un-balanced culture conditions. The highest antioxidant activity was measured in continuous culture at pH 6.5 with an IC50 of 3.44 ± 0.6 µg mL-1, which is close to the IC50 of reference compounds (trolox and α-tocopherol). In addition, the principal component analysis revealed a strong link between the antioxidant activity and the culture method, the photophysiological state and the nitrogen cell quota and C:N ratio of Tetraselmis sp.. These results highlight Tetraselmis sp. as a species of interest for natural antioxidant production and the potential of PAM fluorometry to monitor culture for production of biomass with a high antioxidant activity.

Highlights

► *Tetraselmis* has a higher antioxidant activity in balanced culture in acidic condition. ► CO_2-acidification enhances *Tetraselmis* growth in batch and continuous culture. ► Culture mode and CO_2-acidification have interactive effects on *Tetraselmis* physiology. ► CO_2-acidification with N-limitation induce a decrease of photosynthetic parameters. ► CO_2-acidification and culture mode are effective tools to optimize antioxidant activity.

Keywords : phytoplankton, chlorophyll a fluorescence, non photochemical quenching, photosystem II efficiency, TBARS assay

Abbreviations: Ek: Light saturation index; Fv/Fm: Maximum photosystem II efficiency; IC_{50}:inhibition concentration 50; NPQ: non photochemical quenching; NPQ induc.: non photochemichal quenching induced by the rapid light curves; PBR: photobioreactor; PCA: principal component analysis; PSII: photosystem II; PUFA: poly unsaturated fatty acid; QC: cell carbon content; QN: cell nitrogen content; rETR: maximum relative electron transport rate; RLC: rapid light curve; ROS: reactive oxygen species; RQE: relative quantum efficiency; TBARS: thiobarbituric acid reactive substances

Keywords

phytoplankton; chlorophyll *a* fluorescence; non photochemical quenching; photosystem II efficiency; TBARS assay

1. Introduction

Many natural products isolated from microalgae have attracted attention owing to their broad spectrum of biological activities with health promoting effects. Microalgae produce specific metabolites that can be used in cosmetics and pharmaceuticals; they have bioactive compounds with anti-UV, antibacterial, antifungal, anticancer, anti-inflammatory and/or antioxidant activities (Abad et al., 2008; Assunção et al., 2016; Safafar et al., 2015; Sathasivam and Ki, 2018; Talero et al., 2015; Yuan et al., 2011). In addition, PUFAs (e.g., the omega-3 or -6 fatty acids), pigments (e.g., carotenoids), vitamins, sterols and polysaccharides from microalgae origin are key molecules to develop dietary supplements in human nutrition and to improve animal feed (Aklakur, 2016; A. Catarina Guedes et al., 2011; Ana Catarina Guedes et al., 2011; Plaza et al., 2008).

Microalgae are currently studied as a potential source of natural antioxidant (Assunção et al., 2016; Goiris et al., 2012), as they could replace synthetic antioxidants in the food industry (Batista et al., 2019, 2017; Goiris et al., 2015a), and in the cosmetic, pharmaceutical and nutraceutical industries (A. Catarina Guedes et al., 2011; Sansone and Brunet, 2019). In addition, microalgae biodiversity, productivity and controlled culture conditions offer great potentials to produce a sustainable source of natural antioxidants (A. Catarina Guedes et al., 2011; Mimouni et al., 2012; Wijffels et al., 2010).

Recent screening showed that the antioxidant capacity of microalgae is species specific (Assunção et al., 2016; Coulombier et al., 2020; Custódio et al., 2012; Goiris et al., 2012). A *Tetraselmis* sp. isolated in New Caledonia was identified as a promising species for antioxidant production (Coulombier et al., 2020) owing to its high capacity to inhibit lipid peroxidation, growth rate, and ease of culture. In addition, *Tetraselmis* spp. were also identified for their antioxidant potentials in other screening (Assunção et al., 2016; Custódio et al., 2012; Goiris et al., 2012). This genus is cosmopolitan and can be found in different types of ecosystems, from oceanic to freshwater and hypersaline habitats (Fon-Sing and Borowitzka, 2016; Worden et al., 2004). Many *Tetraselmis* species have a high growth rate and dietary value of interest (e.g., polyunsaturated fatty acids, vitamins) for aquafeed formulation, fish, live feed, and shellfish nutrition (Cerezuela et al., 2012; Hemaiswarya et al., 2011; Ponis et al., 2003; Thinh et al., 1999), thus it is a genus exploited in aquaculture (e.g., *T. chui*, *T. suecica*).

Concerning biotechnological applications, this genus is known to have a high content of bioactive compounds with antioxidant properties such as carotenoids (Ahmed et al., 2014), polyunsaturated fatty acids (Custódio et al., 2012), water soluble polysaccharides (Dogra et al., 2017), phenolic compounds (Farahin et al., 2016; Gam et al., 2020) and vitamins (Brown et al., 1999). Depending on abiotic stressors, microalgae can induce the production of antioxidant molecules to protect its organelles against ROS. This ability can then be used to enhance the production of specific metabolites of interest (Chen et al., 2017; Paliwal et al., 2017). Indeed, nutrient availability (Goiris et al., 2015b), light condition (Coulombier et al., 2020), pH and temperature (A.C. Guedes et al., 2011) are abiotic factors that influence the antioxidant activity and productivity of microalgae. However, few studies assessed the effect of pH or CO_2-induced acidification on microalgae antioxidant activity (A.C. Guedes et al., 2011; Xia et al., 2018). Yet, pH and CO_2-induced acidification affects dissolved inorganic carbon availability, intracellular acid base balance, structural rearrangement of pigment systems, and therefore, may influence growth, carbon assimilation, energy demand to maintain the membrane electrochemical potential and enzyme activity, and intracellular oxidative stress (Goss and Garab, 2001; Kramer et al., 2003; Milligan et al., 2009; Xia et al., 2018). Changes in carbon fixation will also modify the cellular concentration of ATP and NADPH, which in turn may modify photochemical processes and energy dissipation pathways (Takahashi and Murata, 2005). To monitor these photochemical processes (mainly PSII), PAM fluorometry is a commonly used technique based on chlorophyll fluorescence which offers the advantage to be non-invasive (Krause and Weis, 1991; Schreiber et al., 1995) and suitable in microalgal biotechnology to follow large scale culture fitness (Masojídek et al., 2010). This technique measure the light energy emitted from the light harvesting pigments associated with the process of photosynthesis. Briefly light energy absorbed by chlorophyll is either used by the photochemistry (photosynthesis), dissipated as heat (excess of energy) or re-emitted (fluorescence); consequently, by measuring the yield of fluorescence, information about the fitness of the PSII can be estimated (reviewed in Maxwell and Johnson 2000; Consalvey et al. 2005).

Culture conditions are modulating factors to which microalgae cell will respond by adjusting their physiology inducing modifications in growth, photosynthetic parameters, biomass composition and consequently antioxidant activity (Coulombier et al., 2020). In this study, we focus on the effects of pH using CO_2-induced acidification on antioxidant activity and physiological responses of a tropical strain of *Tetraselmis* sp. produced in stirred closed photobioreactor operated in batch and in continuous culture. The overall objectives were to assess the effect of pH and culture mode on growth, photosynthetic parameters, and elemental composition to suggest marker processes of antioxidant activity of *Tetraselmis* sp. and to optimize production of biomass with high antioxidant activity.

2. Materials and methods

2.1. Microalgae culture

The microalgae *Tetraselmis* sp. was isolated from tropical coastal seawater (New Caledonia) (Coulombier et al., 2020). The inoculum was cultured in a 250 mL Erlenmeyer flask with filtered seawater (salinity 35; 0.2 µm) enriched in Conway medium (Walne, 1966). The cultures were exposed to a continuous light intensity of 190 µmol photons m^{-2} s^{-1}, aerated, and gently homogenized daily for 11 days.

2.2. Experimental culture conditions

Continuous and batch cultures were carried out in six 2.5 L stirred closed PBRs made of transparent polymethylmethacrylate (Jauffrais et al., 2013) and operated under the following conditions. The pH was regulated

at 8.5 or 6.5 using automated CO_2 addition and the temperature was kept constant at 25˚C. Temperature and pH conditions were set on an Arduino electronic card and followed with a Raspberry PI computer. The light was continuously provided on one side of the PBR using neon light tubes at 280 µmol photons m^{-2} s^{-1}. A Rushton turbine was used to homogenize the culture medium at 80 rpm min^{-1} and aeration was done by bubbling the culture with 0,2 µm filtered air (1 L min^{-1}) administered using stainless steel tube. Prior inoculation, photobioreactors were sterilized with peroxyacetic acid at 5 ppm for 20 min and rinsed twice with filtered seawater (0.2 µm). The photobioreactors were then inoculated with 125 mL of *Tetraselmis* sp. in filtered seawater enriched with 1 mL L^{-1} of Conway medium (Walne, 1966) to reach an initial concentration of ~1.5 x 10^5 cell mL^{-1}. The dilution rate of continuous cultures was fixed and maintained at 0.5 day^{-1} after the fourth day of culture.

2.3. Photosynthetic parameters

Maximum PSII quantum efficiency (Fv/Fm), rapid light curves (RLC) and Non-Photochemical Quenching (NPQ) were measured daily to provide an overview of the physiological state of *Tetraselmis* sp..

Photosynthetic parameters were measured with a PAM fluorometer (AquaPen-C AP 110-C of Photon Systems Instruments, Czech Republic) with a blue light at 455 nm. A dark adaptation of 1 hour was performed before all the measurements. Fv/Fm was measured with a saturating pulse (3000 µmol photons m^{-2} s^{-1}) and according to the equation 1 (Schreiber et al., 1995):

$$\frac{\mathrm{Fv}}{\mathrm{Fm}} = \frac{\mathrm{Fm}-\mathrm{F0}}{\mathrm{Fm}}, \quad (1)$$

where Fv is variable fluorescence, Fm is maximum fluorescence and F0 is the dark-adapted minimum fluorescence yield.

The rapid light curves were performed with seven incremental irradiances steps (10; 20; 50; 100; 300; 500 and 1000 µmol photons m^{-2} s^{-1}) of 60 s. The phytoplankton physiological parameters were estimated by adjusting the model by Platt et al. (1981) to the experimental data:

$$\mathrm{rETR(I)} = \mathrm{rETRmax} \times \left(1 - \mathrm{e}^{\left(-\alpha \times \frac{\mathrm{I}}{\mathrm{rETRmax}}\right)}\right), \quad (2)$$

where rETRmax (AU) is the maximum relative electron transport rate, alpha (α) the initial slope of the RLC at limiting irradiance and Ek the light saturation index (µmol photons m^{-2} s^{-1}, Ek = rETRmax /α).

NPQ induced by the rapid light curve was calculated according to the Stern Volner NPQ (Williamson et al., 2018) and according to the following equation:

$$\mathrm{NPQ\ induc} = \frac{\mathrm{Fm}-\mathrm{Fm}'}{\mathrm{Fm}'}, \quad (3)$$

where Fm is the maximum fluorescent yield and Fm' the maximum fluorescent yield in actinic light measured at the final RLC step.

NPQ was also measured to assess the recovery of *Tetraselmis* sp. reaction centers to 5 successive light pulses of 60 s with a saturating light of 3 000 µmol photons m^{-2} s^{-1} and 3 dark recovery phases of 88 s.

Using the acquired data, the relative quantum efficiency (Williamson et al., 2018) was calculated to allow comparison and assess recovery dynamics between the two pH conditions:

$$\mathrm{RQE} = \frac{\mathrm{Fq}' \div \mathrm{Fm}'}{\mathrm{Fv} \div \mathrm{Fm}} \times 100, \quad (4)$$

where Fq' is the fluorescence quenched in actinic light (Fq'=Fm'/F'), F' is the fluorescent yield in actinic light.

2.4. Cell growth measurements

Cell growth was followed daily by optical density at 680 nm to measure pigment absorption (chlorophyll *a*), at 800 nm for cell compounds absorption and by counting cells using Malassey hemocytometer under a Leica DM750 microscope (x20) (Leica Microsystems, Germany).

For the batch cultures, using the growth kinetics, a Gompertz model (Equation 5) was fitted to the data (Jauffrais et al., 2017) to assess the maximum growth rate (μmax in day^{-1}), the maximum cell concentration (α expressed in ln(Ct/C0) with Ct and C0 in cell mL^{-1}) and the latency time (λ in day, if present) using MatLab software:

$$F(t)= \alpha \times \exp(-\exp(\mu max \times \exp(1)/\alpha \times (\lambda - t)+1)) , \quad (5)$$

2.5. Sampling for elemental composition, nutrient and antioxidant analyses

For the batch cultures, approximatively 500 mL of culture medium at the beginning of the stationary phase (day 6), and the 2 liters left in late stationary phase (day 18) were collected in each PBR for analyses.

For the continuous cultures, in each PBR and at steady states (i.e., less than 10 % variation of cellular concentration and absorbance during at least three days) 1.25 L was collected daily and over three days for analyses at pH 8.5 and 6.5 respectively.

2.5.1. Elemental analysis

All the equipment used for handling was placed in a furnace at 400 °C for 4 hours to avoid carbon or nitrogen contamination. Aliquots (3, 4 or 5 mL) of culture medium were filtered through pre-combusted glass fiber filters (1.2 μm, Whatman GF/C), dried at 70 °C for 24 h, placed in pre-combusted glass tubes and kept at -20 °C until analysis. The samples were analyzed using an elemental analyzer (SERCON Integra 2, United Kingdom). C:N ratio was calculated by dividing cell carbon content (QC) by cell nitrogen content (QN), to assess nitrogen and carbon status of *Tetraselmis*.

2.5.2. Residual NOX and PO_4 analysis

Approximatively 10 mL of the culture medium were filtered through 0.22 μm syringe filters in a 15 mL falcon tubes and kept at -20 °C until NOx (NO_3+NO_2) and PO_4 analysis. The nutrients were analyzed using a continuous-flow auto-analyzer (AA3 Seal Analytical, United Kingdom).

2.5.3. Antioxidant analysis

The leftover of culture was collected by centrifugation (4500 rpm, 5 min, 4°C), lyophilized and kept at -80°C until extraction. Freeze dried biomass of each sample (50 mg) was first grounded using a pestle and a mortar in the dark at room temperature and suspended in 5 mL of methanol/dichloromethane mixture (50:50 v/v). Then, the extracts were submitted to ultrasound for 10 minutes in an ice bath, and were filtered. This process was repeated until the biomass became colorless, then the extracts were pooled, dried under a steam of nitrogen, and stored at -80°C until antioxidant analysis using lipid peroxidation inhibition assay (TBARS assay). The method adapted from Ahmed et al. (2015) was applied on *Tetraselmis* sp. extracts (Coulombier et al., 2020). Briefly, Fe-ascorbate system was used for oxidation catalysis and linoleic acid as unsaturated fatty acid. Linoleic acid (0.2 mL) was emulsified with Tween 20 (0.4 mL) and phosphate buffer (19.4 mL, 20 mM, pH 7.4). Microalgae dried extracts (1.25 to 200 μg mL^{-1}) or standards (0.03 to 5 μg mL^{-1}) were resuspended in 0.5 mL of ethanol. Then phosphate buffer (0.6 mL), ascorbic acid (0.2 mL, 0.01%), $FeSO_4$ (0.2 mL, 0.01%), and linoleic emulsion (0.5 mL) were added successively and the mixture was incubated at 37°C for 24 h. Subsequently, the oxidation was stopped by mixing 0.4 mL of the extract with

butylated hydroxytoluene (BHT) (0.04 mL, 0.4%) and then 0.44 mL of a solution of thiobarbituric acid (TBA, 0.8%) and trichloroacetic acid (TCA, 4%) was added. The samples were then placed at 100°C for 30 minutes, cooled and centrifuged. The absorbance of the supernatant was then measured at 534 nm. The percentage of inhibition of linoleic acid peroxidation and inhibition concentration (IC_{50}) values were finally calculated (Coulombier et al., 2020).

2.6. Data analysis

Data are expressed as mean ± standard error (SE). After testing for homogeneity of variance and normality (test Kolmogorov-Smirnov), statistical analyses to assess differences among treatments consisted of a one-way or two-way analysis of variance (ANOVA) followed by a Fisher's LSD test, or of Kruskal–Wallis tests. A general linear model was also used to assess differences between treatments, sampling days, and time points generated by repeated NPQ measurement during the NPQ induction by actinic light and dark recovery period. Differences were considered significant at $p < 0.05$.

Additionally, to analyze the potential influence of experimental factors (pH, mode of culture), photophysiological state, nutrients and elemental quota on antioxidant activity (IC_{50}), a Principal Component Analysis (PCA) was performed. All statistical data analyses were carried out using Statgraphics Centurion XV.I (StatPoint Technologies, Inc., United States).

3. Results

3.1. Growth performances

Low pH had a significant influence on the growth (µmax, table 1) of *Tetraselmis* sp. cultured in batch as µmax increased from 1.48 ± 0.16 day^{-1} at pH 8.5 to 2.45 ± 0.18 day^{-1} at pH 6.5, but the latency time (lambda) was slightly higher at low pH (0.44 ± 0.04 day) than at 8.5 (0.18 ± 0.03 day, Table 1). However, the maximum concentration (Cmax) was similar between the two conditions, 7.10 ± 0.02 × 10^6 cells mL^{-1} at pH 8.5 and 7.52 ± 0.02 × 10^6 cells mL^{-1} at pH 6.5 (Table 1 and Fig. 1A and C). In continuous culture at pH 8.5, the steady state was maintained from day 6 to 19 at a cell concentration around 2.3-2.8 × 10^6 cells mL^{-1}. The cell concentration quickly raised to 4.0-5.5 cells mL^{-1} at pH 6.5 but cell concentration and absorbance at steady state were less stable than at pH 8.5 (Fig 1B and D).

3.2. Elemental and residual nutrient analyses

The cell carbon content (QC) was not significantly affected by pH ($F_{1,25}$=2.16, P=0.16); whereas, the mode of culture (batch *vs* continuous culture) had a significant effect on the carbon cell quota ($F_{1,25}$=6.97, P=0.0.15), with lower QC in continuous culture at steady state than in batch culture at stationary phase (Fig. 2A). The cell nitrogen content (QN) was affected by both pH ($F_{1,25}$=6.96, P=0.015) and the mode of culture ($F_{1,25}$=86.91, $P < 0.001$), with higher QN in continuous culture and particularly at pH 8.5 (0.37 ± 0.6 fmol $cell^{-1}$, Fig. 2B). The C:N ratio (Fig. 2C) was thus significantly lower in continuous culture compared to batch culture ($F_{1,25}$=670, $P < 0.001$), but significant differences between pH were only observed in continuous culture ($F_{1,16}$=7.26, P=0.017).

Dissolved NOx and phosphate persisted in the medium throughout all experiments, but NOx concentration was low (0.60 ± 0.07 µmol L^{-1}) indicating N-limitation; whereas, phosphate was always higher than NOx indicating no P-limitation (1.98 ± 0.72 µmol L^{-1}).

3.3. Photosynthetic parameters calculated from rapid light curves (RLCs)

The Maximum PSII quantum efficiency (Fv/Fm, Fig. 3A) in batch cultures started at 0.57 ± 0.01 and quickly reached its highest value after one day of culture at pH 6.5 (0.63 ± 0.02), and after three days of culture at pH 8.5 (0.72 ± 0.00). Then Fv/Fm decreased regularly until the end of the experiment; however, a faster decrease was observed at pH 6.5 (0.38 ± 0.03 at the end) than at pH 8.5 (0.57 ± 0.02). In continuous culture, no differences were observed between the two conditions, once steady state was reached, Fv/Fm remained stable around 0.66 from day 5 to the end of the experiment (Fig. 3B).

Four other photosynthetic parameters were followed over time and calculated from the RLCs: rETRmax (relative maximum electron transport rate), Ek (light saturation coefficient in µmol photons m^{-2} s^{-1}), Alpha (maximum light utilisation coefficient for photosystem II), and NPQ induc (Non-Photochemical Quenching induced during the RLC).

In batch culture, the highest rETRmax (174.4 ± 7.2 AU) was measured at pH 6.5, during the exponential growth phase (Fig. 4A). At pH 8.5 a lower rETRmax was found (163.8 ± 4.1 AU) but a similar trend was observed. In both conditions, rETRmax declined sharply during the linear growth phase and slightly at the stationary phase. Moreover, the decline was significantly steeper at low pH than at pH 8.5 (Fig. 4A and Table 2). In continuous culture, rETRmax was stable and comparable under both conditions and reached values that were similar to the highest one measured in batch culture, 181.9 ± 6.2 and 176.0 ± 12.8 AU at pH 8.5 and 6.5, respectively (Fig. 4B).

In batch culture, Ek increased quickly to reach a maximum during the exponential growth phase followed by bi-phasic decrease, a sharp one during the linear growth phase that slowly stabilized reaching the stationary phase (Fig. 4C). The highest value was reached at day 2 at pH 6.5 (521.7 ± 5.2 µmol photons m^{-2} s^{-1}) and at day 3 at pH 8.5 (478.5 ± 5.7 µmol photons m^{-2} s^{-1}). During the stationary phase Ek decreased to 147.5 ± 16.7 µmol photons m^{-2} s^{-1} and 134.5 ± 11.2 µmol photons m^{-2} s^{-1} at pH 6.5 and 8.5, respectively. Contrary to the batch culture, in continuous culture, Ek stayed stable and similar over time and between conditions, at 570-578 µmol photons m^{-2} s^{-1} (Fig. 4D).

Concerning the maximum light utilization coefficient (alpha, Fig. 4E), it fluctuated between 0.31 ± 0.01 to 0.39 ± 0.01 at pH 8.5, while it declined until 0.18 ± 0.02 from the end of exponential growth phase to the end of the experience at pH 6.5. Similar alpha (0.32-0.30) were calculated in continuous culture at pH 8.5 and 6.5 (Fig. 4F).

Non-Photochemical Quenching induced by the RLCs (NPQ induc, Fig. 4G) was variable during both growth phases but kept increasing at pH 6.5 from day 2 to the end of the experiment (1.68 ± 0.08 on day 18), whereas at pH 8.5, once the stationary phase was reached, the NPQ induc stabilized between 0.92 ± 0.23 - 1.31 ± 0.17. Similarly, to the other calculated photophysiological parameters, no differences were found in continuous culture (0.38-0.31, Fig. 4H).

3.4. Photosynthetic parameters calculated from NPQ data

In batch culture, five days were monitored (Fig. 5) to assess photosynthetic recovery over time. Relative Quantum Efficiency (RQE) was calculated from Non-Photochemical Quenching (NPQ) data measured during the exponential growth phase (day 2), the linear growth phase (day 4) and during early to late stationary phase (days 6, 11, 18). The NPQ and RQE showed significant differences owing to pH, sampling day and time (Fig. 5A, B, D and E and Table 3). However, the magnitude of NPQ induction and recovery was greater at pH 6.5 during stationary phase (day 11 and 18). Furthermore, the RQE decrease was clearly enhanced over the induction period at pH 6.5 at day 11 and 18 (e.g., 60.9 ± 2.8 on day 18, 86.5 ± 3.0 on day 6; Fig. 5E) with an incomplete recovery during the dark recovery period compared to the other condition and sampling days. Interestingly, in continuous culture, NPQ and RQE curves showed similar trends during the induction and dark recovery periods but significant differences were observed (Table 3). The NPQ and RQE had lower values at pH 6.5 than at pH 8.5 (Fig. 5C and F), and the magnitude of the RQE decrease during the induction period was higher and with a slower recovery at pH 6.5.

3.5. Antioxidant activity and elemental and residual nutrient analyses

TBARS assay measures the capacity of *Tetraselmis* sp. extracts to inhibit the chain reaction of lipid peroxidation initiated by the ferrous-ascorbate system. In batch culture, no differences were observed between both pH conditions, but a higher inhibition of lipid peroxidation was observed at low pH in continuous culture. In addition, differences were observed between batch and continuous culture. The best IC_{50} were obtained in continuous culture at pH 6.5, (3.44 ± 0.6 µg mL^{-1}, Fig. 6 and Table 4).

3.6. PCA

Biotic and abiotic factors influencing antioxidant activity (IC_{50}) in all samples collected in batch and continuous culture at pH 6.5 and 8.5 were further analyzed through PCA (Fig. 7). The first and second components accounted for 78% (58% and 20% respectively) of the variability amongst all samples.

The first component, with an Eigen value of 7,5, distinguished high and low IC_{50} values mainly based on the culture method (batch *vs* continuous) and on the photophysiological state of the PSII (Fv/Fm, rETRmax, EK, NPQ induc) and elemental composition (QN and C:N ratio). The second component, with an Eigen value of 2.6, clearly distinguished IC_{50} values owing to the effect of pH, nutrient availability and of the capacity of the PSII to cope with low light (Alpha).

4. Discussion

4.1. Growth performances and fitness

Responses of microalgae to CO_2-induced acidification are likely to be species specific, with potential "winners" and "losers" (Hinga, 2002). *Tetraselmis* F. Stein (1878) is a cosmopolitan genus known to live in a wide variety

of habitats (Fon-Sing and Borowitzka, 2016; Worden et al., 2004). The species studied in this paper came from a coastal and transitional environment and is thus naturally exposed to sudden changes in pH (Wu et al., 2015). In culture, the effect of pH on a *Tetraselmis* sp. growth was studied in batch culture between pH 5.5 and 9.5 by Khatoon et al. (Khatoon et al., 2014). This strain was found to grow in this large range of pH and was thus considered resistant to acidification of its culture medium. However, its optimum for growth, protein, lipid and carbohydrate content was situated between pH 7.5 and 8.5 in batch culture (Khatoon et al., 2014).

In the present study, we studied a *Tetraselmis* sp. cultured under two pH conditions, 6.5 and 8.5, and using two different modes of culture (batch and continuous) to assess the effect of CO_2 acidification on biomass production and fitness, in addition to antioxidant activity. Low pH had a significant influence on growth in batch culture as the acidification of the culture medium enhanced *Tetraselmis* sp. specific growth rate (µmax) by a factor of 1.65. However, the difference of pH only had a negligible effect on the latency time (lambda) and maximum concentration (Cmax), as small or no differences were observed for these parameters. Similarly, in continuous culture the switch from pH 8.5 to 6.5 significantly increased cell concentration by a factor of 1.6; however, looking at the data, the stability (cell concentration, DO) of the culture at steady state showed more variation at pH 6.5 than at pH 8.5. In the present study, the acidification was CO_2-induced. Even though pCO_2 and dissolved inorganic carbon (DIC) were not monitored, chemical changes in seawater carbonate systems are known to occur through CO_2 acidification including higher CO_2:HCO_3^- ratio and increase of DIC (Hinga, 2002); the stimulated growth at pH 6.5 could be explained in two ways : (i) the increase in DIC provided more carbon for *Tetraselmis* for photosynthesis (Mackey et al., 2015; Wu et al., 2010) (ii) the downregulation of energetic cost CO_2 concentrating mechanisms (CCMs) saved energy that may be allocated to support growth (Hu et al., 2017; Xiang et al., 2001). Indeed, marine microalgae assimilate inorganic carbon through the carboxylating enzyme Rubisco, which can only use CO_2 as substrate. However, Rubisco have low affinity to CO_2, and growth can be limited at high pH where dissolved inorganic carbon is mostly find as HCO_3^- and CO_3^{2-}. Therefore, microalgae have developed CCMs to increase CO_2 concentration in the proximity of Rubisco that require energetic investment (Giordano et al., 2005). Microalgae biochemical composition is influenced by these physico-chemical variations of their culture medium, which is then reflected by their physiological state (Brown et al., 1997). This study clearly established that QC of *Tetraselmis* sp. was mainly affected by the mode of culture (batch vs continuous). Even if QC was always higher at pH 8.5 than at pH 6.5, this difference was not significant, whereas QN was affected by both pH decrease by CO_2 acidification and mode of culture, as it was 3-times fold higher in continuous culture than in batch culture, and 1.3-times fold higher in culture at pH 8.5 than at pH 6.5. The C:N ratio was therefore 3.1-times lower in continuous culture compared to batch culture, and a significant difference between pH was only observed in continuous culture. The standard elemental stoichiometry is generally assumed to be 106 C: 16 N: 1 P (Redfield, 1958); however, microalgae in culture have a wide flexibility in their C:N:P ratios depending on which nutrient was limited (Geider and La Roche, 2002). In our study, this ratio was mainly affected by the mode of culture (batch vs continuous), as a higher carbon and a lower nitrogen quotas were observed in batch culture compared to continuous culture. Although nitrate concentration in the culture medium was comparable between stationary phase of the batch culture and steady state of the continuous culture, the continuous supply in nitrate (and other nutrients) in continuous culture induced a higher QN and consequently a lower C:N. It results in typical C:N ratios of N-limited microalgae (>15) in batch culture and N-non limited microalgae (<10) in continuous culture (Rios et al., 1998). This effect on C:N ratio is a common observation owing to nitrate deficiency: a decrease in N products,

such as chlorophyll and proteins (up to 25%), and an increase of carbon storage products, such as carbohydrates and eventually lipids (up to 50-60% AFDW for both) (Jiang et al., 2012; Li et al., 2011).

The responses to CO_2-induced acidification also lead to different photophysiological fitness. The observations can be modulated depending on the mode of culture used to produce *Tetraselmis* sp.. Indeed, differences due to CO_2 acidification were enhanced in batch culture compared to continuous culture. Under both pH conditions in the batch culture, Fv/Fm increased during the exponential growth phase to stabilize and decrease once the stationary phase was reached. The Fv/Fm values are similar to those previously measured for eukaryotic microalgae cultivated in similar culture conditions (Barnett et al., 2015; Goiris et al., 2015b; Jauffrais et al., 2016; Menguy et al., 2020). Yet, Fv/Fm reached a lower maximum value and decreased faster at pH 6.5 compared to pH 8.5. However, this difference between quantum yields was not observed in continuous culture, where Fv/Fm remained stable over time independently of pH. The maximum quantum efficiency of the PSII reflects photochemical processes that depend upon chloroplast reactions that use ATP and reductants provided by photosynthesis. It may therefore be sensitive to cell energy metabolism and interactions between carbon and nitrogen assimilation. However, the use of Fv/Fm to monitor a photophysiological stress may be controversial, e.g., to monitor nutrient stress in balanced systems (Napoleon et al., 2013). Nevertheless, Fv/Fm is still a useful indicator of environmental or nutritional stress of microalgae grown in batch cultures (Beardall et al., 2001; Gordillo et al., 2001; Jauffrais et al., 2016) and is commonly used as an indicator to asses pH and/or CO_2 effect on photochemical processes of phytoplankton (Touloupakis et al., 2016; Wu et al., 2015). Our study seems to confirm these differences between balanced and unbalanced modes of culture. However, it also highlights that over time and under stable and balanced conditions (e.g., nutrients), *Tetraselmis* sp. is able to adapt and maintain its maximum quantum yield under CO_2-acidified culture medium.

Similarly to Fv/Fm, the photosynthetic parameters calculated from the RLCs (rETRmax, Ek, Alpha and NPQ induc) highlight differences between the two-pH conditions only in batch culture whereas no difference was observed in continuous culture. At pH 6.5 in batch culture, the lower Fv/Fm, rETRmax and alpha, and the higher NPQ induc reflect an increased photo-stress and corresponding suppression of photochemistry. This photo-stress might be related, in batch culture, to low pH stress coupled with N-limitation. Indeed, it is known that N-limitation gradually causes a decrease in photosynthetic pigments (Geider et al., 1993) the decline of PSII maximum quantum yield (Fv/Fm) (Berges et al., 1996; Cleveland and Perry, 1987; Jauffrais et al., 2016) and the gradual inactivation of the protein D1 in PSII reaction centers owing to the reduced amount of N compounds to synthesize it. Protein D1 is an essential component in the electron transport chain and its turnover rate is frequently the limiting factor in PSII repair rates (reviewed in Campbell and Tyystjarvi(Campbell and Tyystjarvi, 2012). In addition, N-limitation may reduce internal pH regulation mechanisms, to balance the external pH decrease, inducing acidification of intracellular compartments such as cytoplasm and chloroplast's stroma that could exerts numerous effects, in particular Rubisco inhibition (Miyachi et al., 2003; Solovchenko and Khozin-Goldberg, 2013). It could explain the greater Fv/Fm, rETRmax and alpha decrease and the NPQ induc increase at pH 6.5 compared to pH 8.5. (Ptushenko and Solovchenko, 2016).

Microalgae modulate their NPQ to tolerate and prevent long-lasting damage to photosynthetic components, owing to high or fluctuating levels of light, by dissipating the excess absorbed energy as heat (Lavaud et al., 2007; Lavaud and Kroth, 2006; Wu et al., 2012). However, excessive irradiance, either long or sudden, can reduce NPQ efficiency, leading to damage of the protein D1, decline in photosynthetic efficiency, chronic photoinhibition and

production of ROS (Franklin and Forster, 1997). The studied species was isolated in a costal and tropical area (New Caledonia) where photosynthetic active radiations are often above 2000 µmol photons m-2 s-1. In addition, this strain is not known to be highly sensitive to high light intensity (Coulombier et al., 2020). However, CO_2-induced acidification is known to influence NPQ and particularly to increase NPQ at high light level (Gao et al., 2012). NPQ and the relative quantum efficiency (RQE, e.i. the quantum efficiency as a proportion of Fv/Fm) were thus monitored under actinic light induction and dark recovery phases to allow comparison of induction and recovery dynamics between the two pH conditions and culture modes (Williamson et al., 2018).

In batch culture, both NPQ and RQE showed significant differences owing to pH, sampling day and time. However, the magnitude of NPQ induction and recovery was greater at pH 6.5 during stationary phase and the RQE decrease was clearly enhanced over the induction period with an incomplete recovery during the dark recovery period. These results in batch confirm the photo-stress triggered by both low pH and N-limitation.

In continuous culture, NPQ and RQE curves showed similar trends during the induction and dark recovery periods but significant differences were observed. The magnitude of the RQE decrease during the induction period was higher and with a slower recovery at pH 6.5. It highlights that independently of N-limitation and culture mode, the CO_2-induced acidification to pH 6.5 induced a higher sensitivity to photo-stress in *Tetraselmis* sp.. At pH 6.5, there is more dissolve CO_2 in the medium which may induce an intracellular acidification due to entry of CO_2 into the cell. Low internal pH is known to provoke inhibition of Rubisco which reduces consumption of NADPH and ATP and decrease electron sink from the photosynthetic electron transport chain (Miyachi et al., 2003; Ptushenko and Solovchenko, 2016). In addition, NPQ (xanthophyll cycle and protonation of PsbS protein) is known to be triggered by acidification of the thylakoid lumen when chloroplasts are illuminated (Goss and Garab, 2001; Kramer et al., 2003). It could explain the negative impact of CO_2 acidification on photophysiological parameters.

4.2. Antioxidant activity

All aerobic organisms have to deal with ROS. Furthermore, microalgae possess chloroplasts where ROS are formed via energy transfer from chlorophyll or via electron transfer. Indeed, ROS concentration controls the PSII activity and therefore photosynthesis and photoprotective responses (Foyer and Shigeoka, 2011; Smerilli et al., 2019, 2016). Environmental stress, such as CO_2-induced acidification (Bautista-Chamizo et al., 2018) or N-starvation (Çakmak et al., 2015; Chokshi et al., 2017; Zhang et al., 2013) are known to induce additional ROS production An efficient intracellular network composed of enzymes and antioxidant molecules is thus required to balance and adjust intracellular ROS concentration (Foyer and Noctor, 2005; Mittler, 2002). In addition, recent works on microalgae showed that a strong link exists between antioxidant molecules and photo-protective mechanisms (xanthophyll cycle and NPQ) in response to light (Cartaxana et al., 2013; Smerilli et al., 2019, 2016). In light of this data, we analyzed the effects of CO_2-induced acidification and mode of culture on antioxidant activity of *Tetraselmis*.

The bio-complexity of antioxidant molecules in microalgae requires the use of specific antioxidant assays with different mechanisms of action to evaluate their right antioxidant potential (Carocho and Ferreira, 2013; Coulombier et al., 2020; Prior et al., 2005; Rodriguez-Amaya, 2010). Different tests (e.g., TBARS, ORAC, DPPH and ABTS) were used in our previous study (Coulombier et al., 2020), with variable sensitiveness to different antioxidant compounds (e.g., PUFAs, carotenoids, phenolic compounds). We found that the TBARS assay, which measures the capacity of an antioxidant to inhibit the chain reaction of lipid peroxidation, was the best assay to assess the antioxidant activity of *Tetraselmis* sp. extracts (Coulombier et al., 2020). In this study, the best IC_{50}

were obtained with reference compounds trolox (0.24 µg mL-1) and α-tocopherol (1.30 µg mL-1); however, the IC_{50} found in continuous culture at low pH (3.44 ± 0.6 µg mL-1) was close to the IC_{50} of these reference compounds. In addition, antioxidant activity was improved by a factor of 4.5 compared to our previous results where IC_{50} of *Tetraselmis* sp. extracts were equal to 15.43 and 22.77 µg mL−1 under low and high light respectively. Furthermore, the capacity of *Tetraselmis* sp. extracts to inhibit lipid peroxidation was more than seven time higher than other microalgal extracts, e.g., *Nitzschia* sp. (24.63 µg mL−1), and *Nephroselmis* sp. (31.40 µg mL−1) (Coulombier et al., 2020).

Considering TBARS assay, our previous results showed that carotenoids did not contribute to the antioxidant activity measured (Coulombier et al., 2020). Lipid peroxidation is known to be inhibited by phenolic compounds (Cho et al., 2003; Niki et al., 2005; Salah et al., 1995), α-tocopherol, fatty acid compounds (Henry et al., 2002; Richard et al., 2008) and sterols (Gordon and Magos, 1983; Yoshida and Niki, 2003). However, phenolic compounds and α-tocopherol are probably not the molecules involved in *Tetraselmis* sp. extract as no activities were found using DPPH and ABTS assays (Coulombier et al., 2020). Since *Tetraselmis* is a genus known for its high content in PUFAs (Brown et al., 1997) and sterols (Patterson et al., 1993) and as we used a mixture of MeOH/DCM similar to the one used for lipid extraction (Cequier-Sánchez et al., 2008), a high concentration of sterols or PUFAs in the extracts could explain our results on antioxidant activities (Coulombier et al., 2020; Custódio et al., 2012; Lv et al., 2015). However, it needs to be confirmed by a bio-guided fractionation of the extracts to identify antioxidant compound(s). Nevertheless, microalgae whole biomass or crude extract are often use for nutraceuticals or aquaculture preparations, thus it is necessary to optimize global antioxidant activity of the biomass instead of a specific antioxidant molecule.

We analyzed the potential influence of experimental factors (pH, mode of culture), photophysiological state, nutrients and elemental quota on antioxidant activity (IC_{50}) with a PCA, the objective being to improve and monitor culture conditions for producing biomass with high antioxidant activity. The first component distinguished high and low IC_{50} values mainly based on the culture method (batch *vs* continuous) and on the photophysiological state of the PSII (Fv/Fm, rETRmax, EK, and NPQ) and elemental composition (high QN and low C:N ratio); whereas, the second component clearly distinguished IC_{50} values owing to the effect of pH, of nutrient availability and of the capacity of the PSII to cope with low light (Alpha).

In addition to pH and culture mode, results revealed a strong link between the state of the PSII and the antioxidant activity of *Tetraselmis* sp.. The availability in dissolved N and P is also a key factor to improve antioxidant activity of *Tetraselmis* sp..

To sum up, a higher antioxidant activity (low IC_{50}) is observed in continuous culture where nutrients availability is balanced and where the microalgae are in great photophysiological state. However, these conclusions need to be taken with some care and further studies are necessary to clearly link PSII state with antioxidant molecules and activities to improve the use of PAM fluorometry to monitor microalgae for producing biomass with high antioxidant activity.

5. Conclusions

This study provides evidence of the interactive effects of CO_2-induce acidification and nutrient availability on growth, photophysiological state of the PSII and antioxidant activity of *Tetraselmis* sp.. It also highlights the great potential of *Tetraselmis* sp. as an alternative source of natural antioxidant, as well as on the role of pH and nutrients

as effective tools to enhance the production of biomass with high antioxidant activity. In addition, it suggests that PAM fluorometry might be used to monitor microalgae culture for antioxidant-rich biomass production.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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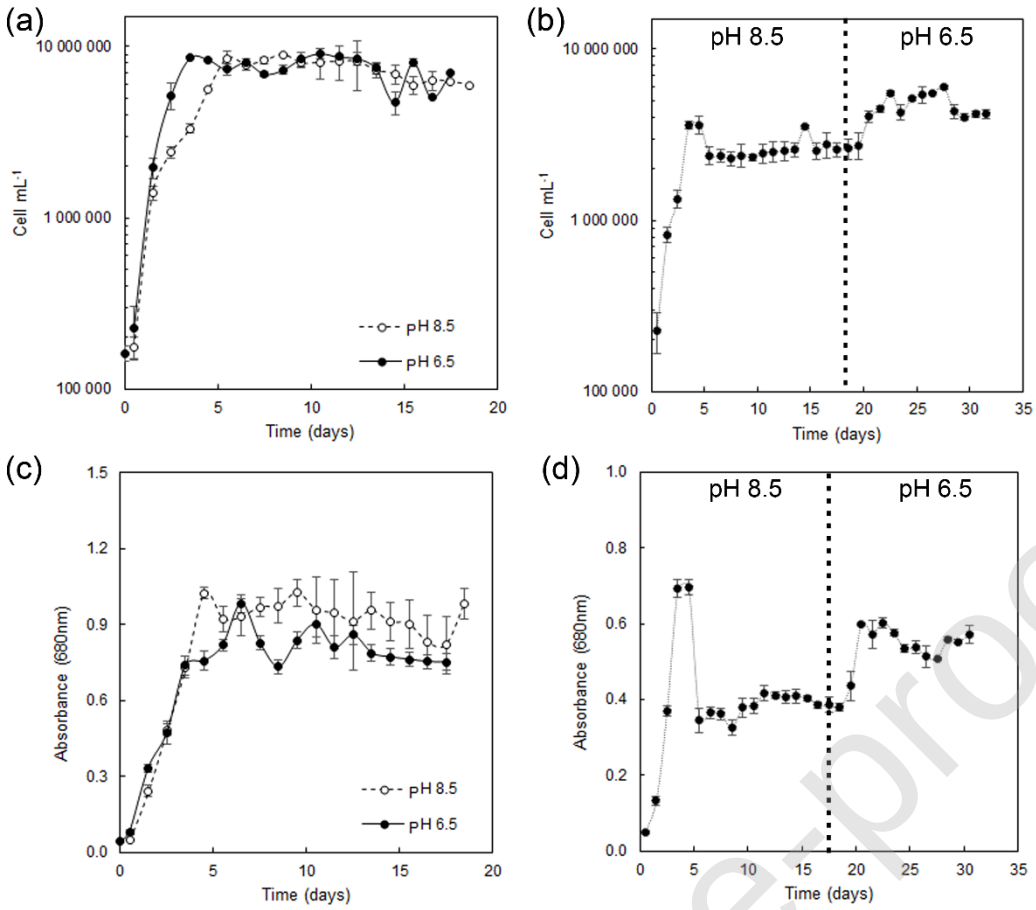

Fig. 1 (a), (b) Cell concentration in Cell mL-1; **(c), (d)** Absorbance at 680 nm of *Tetraselmis* sp. cultured in batch (left panels) and continuous (right panels) cultures in PBRs at pH 8.5 and 6.5 over time (days). Data are expressed as mean ± standard error (SE, n = 3)

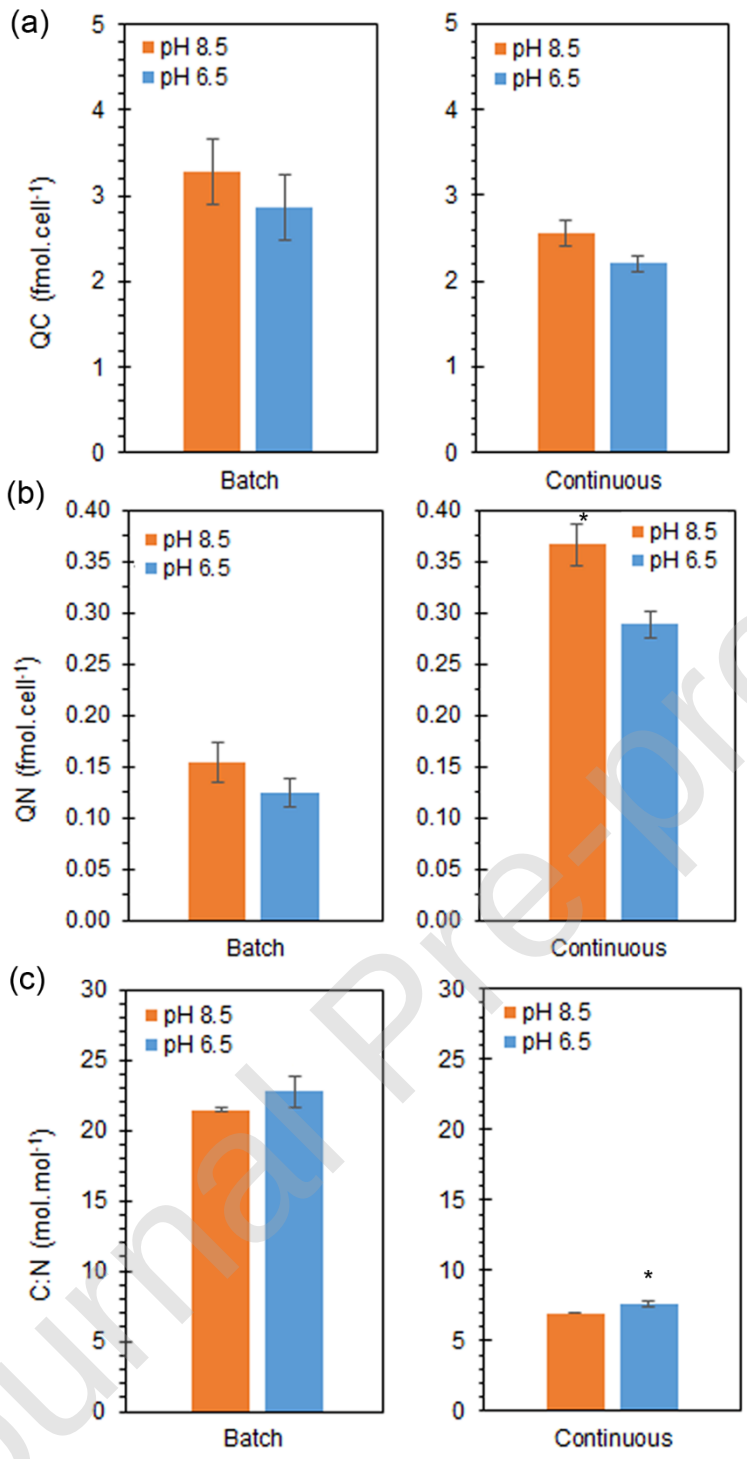

Fig. 2 (a) Carbon cell quota (fmol cell^{-1}) QC; **(b)** Nitrogen cell quota QN (fmol cell^{-1}); **(c)** C:N (Carbon:Nitrogen) ratio of *Tetraselmis* sp. in batch culture at stationary phase and in continuous culture at steady state at pH 8.5 and 6.5. * indicates significant differences between conditions (P-value<0.05). Data are expressed as mean ± standard error (SE, n = 6 in batch and 12 in continuous culture)

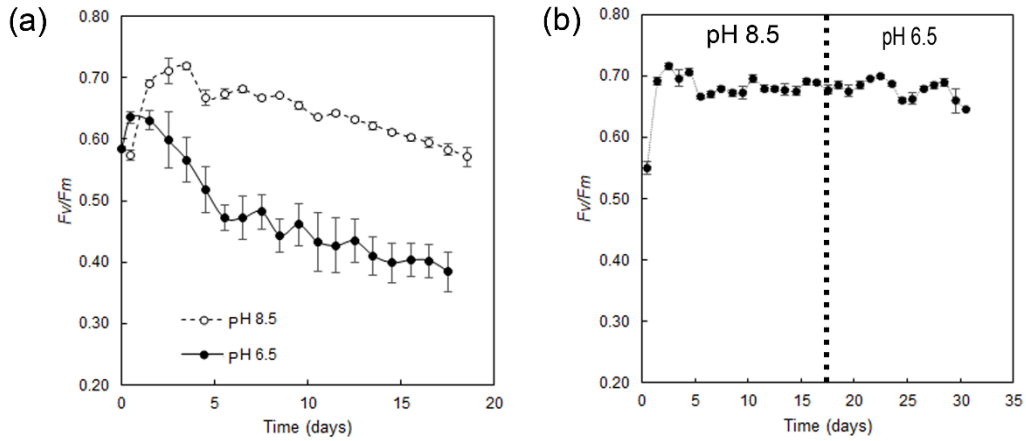

Fig. 3 (a), (b) Maximum quantum light utilization efficiency of photosystem II (PSII, Fv/Fm) of *Tetraselmis* sp. in batch (left panels) and continuous (right panels) cultures in PBRs at pH 8.5 and 6.5 over time (days). Data are expressed as mean ± standard error (SE, n = 3)

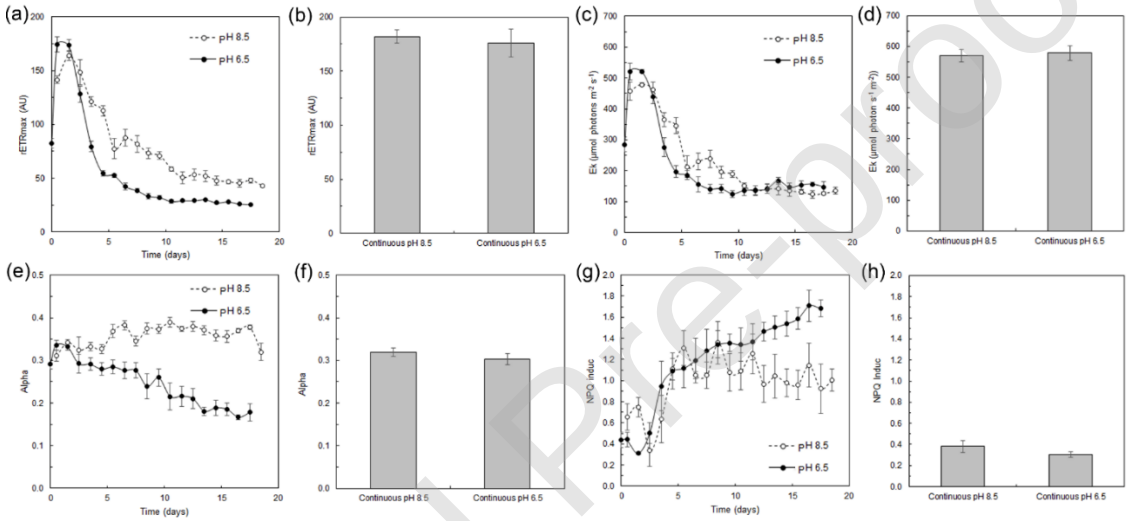

Fig. 4 Photosynthetic parameters obtained from rapid light curves carried out on *Tetraselmis* sp. in batch (a, c, e, g) and continuous (b, d, f, h) cultures in PBRs at pH 8.5 and 6.5. (**a), (b)** Maximum electron transport rate rETRmax in AU; (**c), (d).** light saturation coefficient Ek in µmol photons m^{-2} s^{-1}; (**e), (f)** Maximum light utilization coefficient for photosystem II Alpha; (**g), (h).**Non-Photochemical Quenching induced during RLC NPQ induc. Data are expressed as mean ± standard error (SE, n = 3)

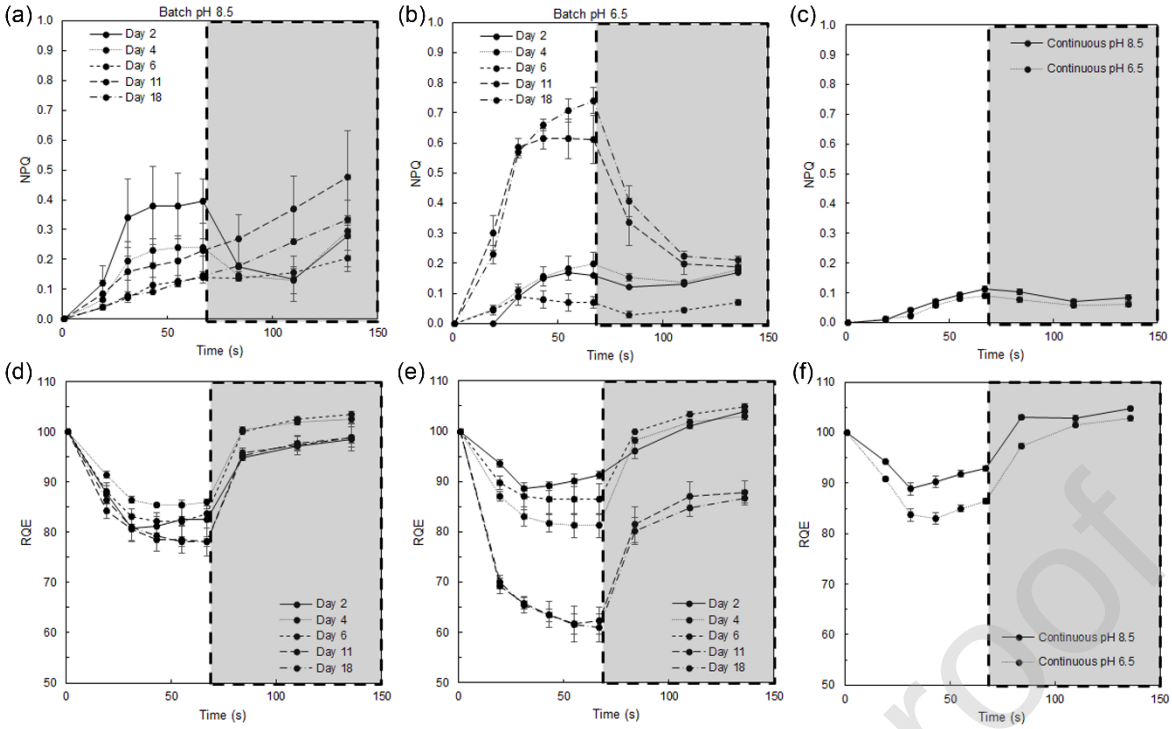

Fig. 5 (a), (b), (c) Non-Photochemical Quenching (NPQ) over the induction (white background) and dark recovery (grey background) **(d), (e), (f)** Relative Quantum Efficiency (RQE) of *Tetraselmis* sp. in batch (days 2, 4, 6, 11, 18) and continuous cultures (the two steady states) in PBRs at pH 6.5 and 8.5. Data are expressed as mean ± standard error (SE, n = 3)

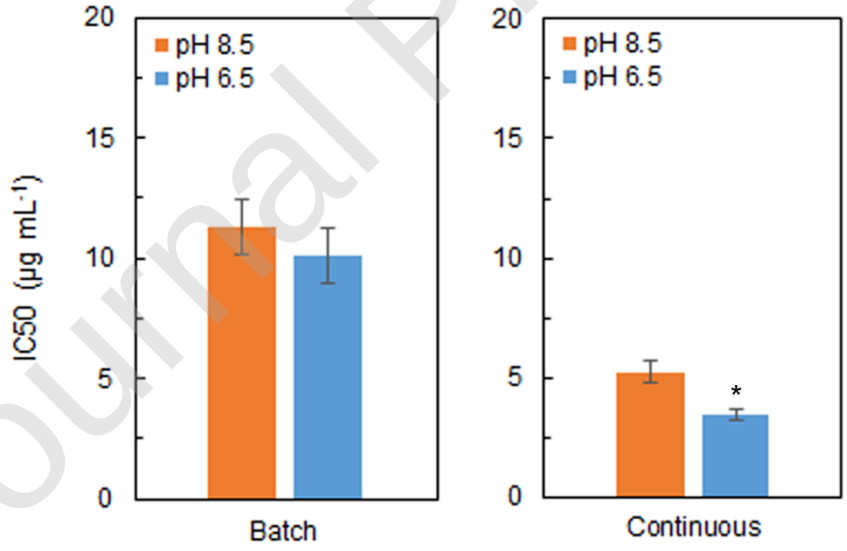

Fig. 6 Evolution of the antioxidant activities (IC_{50}, µg mL^{-1}) of *Tetraselmis* sp. extracts in batch culture at stationary phase and in continuous culture at steady state at pH 8.5 and 6.5. * indicates significant differences between conditions (P-value<0.05). Data are expressed as mean ± standard error (SE, n = 6 in batch and 12 in continuous culture). Reference compounds trolox (IC_{50} = 0.24 µg mL^{-1}) and α-tocopherol (IC_{50} = 1.30 µg mL^{-1})

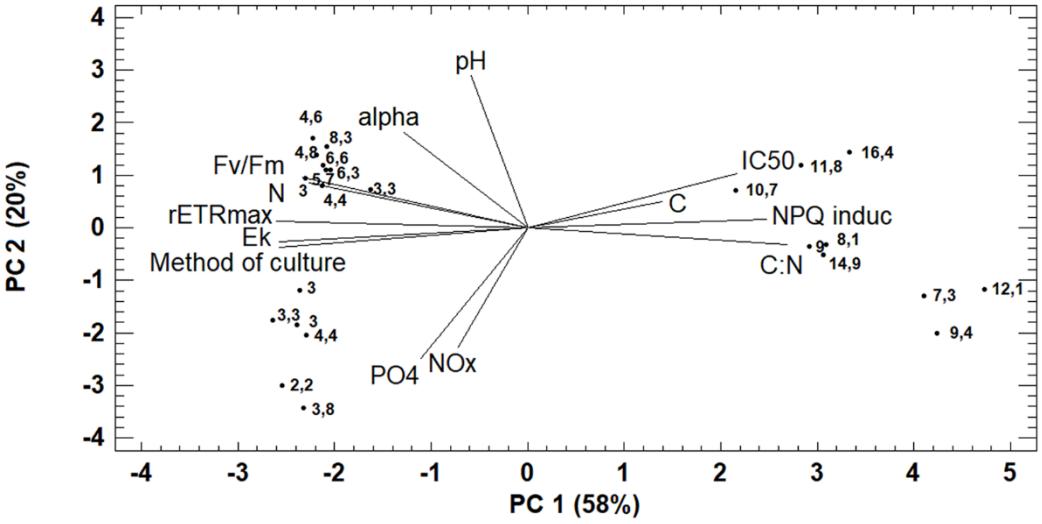

Fig. 7 Results of the first and second component analysis influencing the antioxidant activity (IC_{50}, black dot with their associated values) of *Tetraselmis* sp.. Fv/Fm (Maximum quantum light utilization efficiency of Photosystem II), rETRmax (relative maximum electron transport rate), Ek (light saturation coefficient in µmol photons m^{-2} s^{-1}), alpha (maximum light utilisation coefficient for photosystem II), NPQ induc (Non-Photochemical Quenching induced during RLC), NOX (nitrate + nitrite) at sampling time, N and C (nitrogen and carbon quotas), C:N (carbon:nitrogen ratio)

Table 1 Cell growth parameters, maximum growth rate (µmax), latency time (lambda) and maximum cell concentration (Cmax) of *Tetraselmis* sp. cultured in PBRs in batch culture at pH 8.5 and 6.5. Data are calculated using a Gompertz model and expressed as mean ± standard error (SE, n = 3). Differences were considered significant at $p < 0.05$

Batch culture	pH 8.5	pH 6.5	F-ratio	P (α=0.05)
µmax (day^{-1})	1.48 ± 0.16	2.45 ± 0.18	15.74	**0.02**
Lambda (day)	0.18 ± 0.03	0.44 ± 0.04	22.57	**0.01**
Cmax ($\times 10^6$ cells mL^{-1})	7.10 ± 0.83	7.51 ± 0.21	2.11	0.22
Adjusted R^2	0.97 ± 0.01	0.97 ± 0.01	-	-

Table 2 Results of the statistical analysis on photosynthetic parameters obtained from rapid light curves carried out on *Tetraselmis* sp. in batch (General linear model) and continuous cultures (Kruskal Wallis test) in PBRs at pH 8.5 and 6.5. Fv/Fm, Maximum quantum light utilization efficiency of Photosystem II (PSII); rETRmax, maximum electron transport rate in AU; Ek**,** light saturation coefficient in µmol photons m^{-2} s^{-1}; Alpha**,** maximum light utilisation coefficient for photosystem II; NPQ induc**,** Non-Photochemical Quenching induced during RLC. Values are significantly different when P<0.05.

Batch culture	pH F-ratio	pH P-value	Time F-ratio	Time P-value
Fv/Fm	283.84	<0.001	6.63	<0.001
rETRmax	99.23	<0.001	61.30	<0.001
alpha	171.3	<0.001	1.47	0.11
Ek	6.44	<0.05	50.30	<0.001
NPQ induc	16.11	<0.001	6.87	<0.001
Continuous culture	pH 8.5	pH 6.5	P-value	
Fv/Fm	0.71 ± 0.01	0.67 ± 0.03	<0.05	
rETRmax	181.90 ± 6.20	176.02 ± 12.80	0.56	
alpha	0.32 ± 0.01	0.30 ± 0.01	0.56	
Ek	570.62 ± 19.83	578.62 ± 24.47	0.70	
NPQ induc	0.38 ± 0.06	0.31 ± 0.03	0.72	

Table 3 Results of the general linear model analysis on Non-Photochemical Quenching (NPQ) and Relative Quantum Efficiency (RQE) of *Tetraselmis* sp. in batch (sampling day 2, 4, 6, 11 and 18) and continuous cultures (steady states) in PBRs at pH 6.5 and 8.5 over the induction and dark recovery (Time). Values are significantly different when P<0.05

	NPQ		RQE	
Batch culture	F-ratio	P-value	F-ratio	P-value
A: pH	8.41	<0.01	27.57	<0.001
B: Sampling Day	31.1	<0.001	42.37	<0.001
C: Time	19.17	<0.001	60.62	<0.001
A*B	27.95	<0.001	22.66	<0.001
A*C	5.38	<0.001	0.58	0.79
A*B*C	3.26	<0.001	0.48	0.97
	NPQ		RQE	
Continuous culture	F-ratio	P-value	F-ratio	P-value
A: pH	17.3	<0.01	22.91	<0.01
B: Time	47.68	<0.001	28.98	<0.001

Table 4 Results of the multifactorial and single ANOVAs to assess the effect of pH, sampling day (early and late stationary phase), and culture systems (batch *vs* continuous cultures) on antioxidant activities (IC_{50}, µg mL^{-1}) of *Tetraselmis* sp.. Values are significantly different when $P<0.05$

	IC_{50}	
Batch culture	F-ratio	P-value
A: Sampling Day	0.53	0.49
B: pH	0.52	0.49
A*B	1.8	0.22
	IC_{50}	
Continuous culture	F-ratio	P-value
pH	6.65	0.02
	IC_{50}	
Batch *vs* continuous culture	F-ratio	P-value
A: Culture system	58.62	<0.001
B: pH	3.4	0.08
A*B	0.21	0.65