Screening for a low-cost *Haematococcus pluvialis* medium reveals an unexpected impact of a low N:P ratio on vegetative growth

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Abstract

Haematococcus pluvialis is the current better source of natural astaxanthin, a high-value carotenoid. Traditionally, the production process of astaxanthin by this algae is achieved by a two-stage system: during the first stage, vegetative "green" cells are produced and then converted, in the second stage, into cysts that accumulate astaxanthin. In this work, a medium screening strategy based on the mixing of a 3-component hydroponic fertilizer was applied to identify a new formulation optimized for the vegetative stage. A maximal and high cell density of 2 x 10^6 cells mL^{-1} was obtained in a medium containing a high level of phosphate relative to nitrate, resulting in a N:P ratio much lower than commonly used media for *H. pluvialis*. In this medium, cells remained at the vegetative and motile stage during a prolonged period of time. Both high cell density culture and motile stage persistence was proved to be related to the N:P feature of this medium. We conclude that the macrozoid stage of *H. pluvialis* is favored under high-P and low-N supply and that low-cost hydroponic fertilizers can be successfully used for achieving high density cultures of vegetative cells of *H. pluvialis*.

Keywords *Haematococcus pluvialis*, Culture media optimization, Phosphate supply, Nutrient-induced fluorescence transients

Introduction

Haematococcus pluvialis Flotow (Chlorophyceae) is a unicellular green alga, which is in a bi-flagellated motile form under optimal environmental conditions. In response to adverse conditions, it enters a resting stage and transforms into cysts, which are enlarged cells with a thick and resistant cell-wall and accumulate large amounts of carotenoids, especially astaxanthin.

Astaxanthin (3-3'-dihydroxy-β,β'-carotene-4,4'-dione) is a high-value secondary carotenoid traditionally used as a red dye in aquaculture feed industry. Because of its high antioxidant potential, greater than β-carotene and tocopherol (Naguib, 2000), this ketocarotenoid has also received an increased interest for clinical applications: natural astaxanthin has been proved useful for cancer treatment (Palozza et al., 2009) and to have an anti-inflammatory action in cardiovascular disease (Fassett and Coombes, 2009). Today, most of commercially available astaxanthin is a synthetic product. However, the growing demand for natural foods and the high cost of chemical synthesis has encouraged the research on production from natural sources (Lorenz and Cysewski, 2000). Among the few micro-organisms able to synthesize astaxanthin, *H. pluvialis* is one that accumulates the most, up to 4 % of its biomass (Aflalo et al., 2007).

Accumulation of astaxanthin in *H. pluvialis* occurs in response to photo-oxidative stress, i.e. when energy input exceeds growth capacity (for review see Lemoine and Schoefs, 2010). Astaxanthin synthesis has long been associated with growth arrest and cyst development. Although the feasibility of continuous production of astaxanthin by vegetative motile cells (macrozoids) has been recently demonstrated (Del Río et al., 2008), the most common production process consists in separating the biomass production phase and the astaxanthin accumulation phase. Finding optimal culture conditions for each of these 2 phases has been the topic of numerous studies (Borowitzka et al., 1991; Cifuentes et al., 2003; Domínguez-Bocanegra et al., 2004; Fábregas et al., 2000; García-Malea et al., 2005; Harker et al.,

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1995; Choi et al., 2002). Even if the exact influence or efficiency of some specific factors on growth - N-source (Borowitzka et al., 1991; Cifuentes et al., 2003), acetate addition (Cifuentes et al., 2003; Jeon et al., 2006), light level (Borowitzka et al., 1991; García-Malea et al., 2005; Harker et al., 1995) - and on astaxanthin induction - salt stress, phosphate deficiency (Borowitzka et al., 1991; Choi et al., 2002), light requirement (Cifuentes et al., 2003; Choi et al., 2002; García-Malea et al., 2005) - are still unclear or debated, the general rule governing the green and red stages is widely accepted: a low C:N ratio is favorable to the production of green biomass and, conversely, the production of astaxanthin occurs preferentially when C:N is high (Kakizono et al., 1992).

In practice, low C:N ratio for green biomass production is generally achieved by growing the cells at low light in media containing saturating levels of nitrate. The Bold Basal Medium (BBM, Nichols and Bold, 1969) has been often used in its initial formulation or modified to contain up to 4-fold more nitrate (Domínguez-Bocanegra et al., 2004; Fábregas et al., 2000). Since biomass accumulation is the major bottleneck in the 2-stage process of astaxanthin production, further optimizations of the growing medium have been undertaken (Cifuentes et al., 2003; Fábregas et al., 2000; Harker et al., 1995; Gong and Chen, 1997; Sarada et al., 2002).

A common approach to determine the factors for optimal biomass production has been to start from a known medium and change relative concentrations of different macro-elements and micro-elements in the medium. In an attempt to optimize the elemental composition of an *Haematococcus* growing medium, Fábregas et al. (2000) evaluated the contribution of 18 components on the final yield of vegetative biomass using a single-variable optimization strategy. In a semi-continuous culture, the steady-state cell density they obtained with their optimized medium (OHM) was three times higher than with the often used BBM. However, as noticed by the authors, this "one-factor-at-a-time" strategy probably lacks to identify positive or negative interactions between nutrients or between nutrient availability and other environmental conditions.

In a comparative study, Dalay et al. (2007) observed, comparing 9 common medium formulations, that biomass accumulation was maximal in a medium prepared with a common agricultural fertilizer. As a starting point of our study, we looked for a low-cost and optimized growing medium for *H. pluvialis* based on the use of a commercial fertilizer. Our optimization strategy relied on the use of a 3-component hydroponic fertilizer which allowed to vary the composition of the media by using different ratios of the 3 core solutions. By screening 18 different combinations for vegetative high density cultures, we selected the best-performing formulation and compared growth, nutrients (NO_3, PO_4) uptake and carotenoid accumulation in batch culture of *H. pluvialis* in this medium and two other common formulations. The relationship between the unusual N and P content of the identified medium and growth of vegetative cells were further investigated.

Table 1: Composition of the Floraseries components
Nutrient concentrations (guaranteed minimum concentrations) in % (w/v) as described by the producer (GHE, Fleurance, France) for each of the 3 components of the FloraSeries fertilizer. M: FloraMicro, G: FloraGro, B: FloraBloom.

Constituents	FloraSeries components M	G	B
Nitrogen	5.0	3.0	
...Ammonium N	1.0	1.0	
...Nitrate N	4.0	2.0	
Phosphate (as P_2O_5)		1.0	5.0
Potassium (as K_2O)	1.3	7.0	4.0
Magnesium		0.8	3.0
Sulfur		1.0	5.0
Boron	0.010		
Calcium	7.000		
Copper	0.010		
Iron	0.120		
Manganese	0.040		
Molybdenum	0.004		
Zinc	0.015		

Materials and Methods

Algal strain and culture conditions *Haematococcus pluvialis* (strain 34/1D) was obtained from the Culture Collection of Algae and Protozoa of the Center for Hydrology and Ecology, Ambleside, UK. Stock cultures were grown in 100 mL of modified BBM3N in 250 mL cell culture flask (Cellstar Suspension Culture Flasks, Greiner Bio-One, Belgium). Modified BBM3N was prepared from a concentrated Bold modified Basal Freshwater Nutrient solution (Sigma-Aldrich, Belgium) to which $NaNO_3$ was added up to 8.82 mM. Flasks were placed horizontally to increase the gas exchange surface and optimize exposition to light. They were gently shaken twice-a-day. Cultures were not supplied with an extra source of CO_2. Temperature was maintained at 25°C, cultures were continuously illuminated by Phillips Master TL5 HO 54W/827 fluorescent lamps. Light intensity was either 30 μmol m^{-2} s^{-1} (low-light) or 150 μmol m^{-2} s^{-1} (high-light).

Media used in the screening were prepared by mixing 3 components of the Floraseries hydroponic fertilizer (GHE, Fleurance, France, guaranteed minimum concentrations, see Table 1). Cells from stock culture were collected at the vegetative stage, cen-

trifuged (3 min, ± 3000 x g, room temperature) and resuspended in 50 mL flasks (Cellstar Suspension Culture Flasks, Greiner Bio-One, Belgium) at the specified density in 10 mL of each of the media formulations tested.

Analytical procedures Cell densities were established by counting under the microscope, using an improved Neubauer hematocytometer. For pigments analysis, cells were collected by centrifuging culture aliquots (3 min, 16000 x g, room temperature). The supernatant was discarded and the pellet was homogenized in 100 % methanol. The extraction procedure was repeated until cell debris was almost colorless. Chlorophylls and total carotenoids were quantified with a UV-VIS spectrophotometer (PerkinElmer UV-Vis spectrophotometer Lambda 20) by recording absorbance at 470, 652 and 665 nm and using the equations of Lichtenthaler (1987).

Investigations of Nutrient Induced Fluorescence Transients (NIFT) involved the recording of chlorophyll *a* fluorescence by Pulse Modulated Fluorimeter (PAM, MFMS, Hansatech). 2 mL of culture sample were placed into a glass cuvette connected to the PAM and containing a magnetic stirrer. The actinic light (λ=650 nm) was set at 30 μmol m^{-2} s^{-1}. After the sample had been left to stabilize for 10 min, H_2O (control), 500 μM of either $NaNO_3$ or K_2HPO_4 were added to the cuvette. Any significant change in fluorescence measured at 680 nm (the analytic modulated light was provided by light-emitting diodes with a central emission wavelength at 580 nm and a photon flux of 0.5 μmol m^{-2} s^{-1}) following nutrient addition was considered as a NIFT.

Nitrate in media was assayed by the colorimetric reaction with salicylic acid as described by Cataldo et al. (1975). Briefly, 20 μL of diluted sampled medium were incubated with 2.5 % (w/v) salicylic acid in H_2SO_4 98 % for 20 minutes before neutralization with 500 μL NaOH 3.8 M. Concentration in NO_3 was then assayed by measuring absorbance at 405 nm with a spectrophotometer (Victor X3, Multilabel Plate Reader, Perkin-Elmer) and comparison with a $NaNO_3$ standard curve.

Phosphate was assayed by reaction with ascorbic acid as described by Chen et al. (1956). Briefly, 50 μL of diluted sampled medium were mixed with 50μL of a freshly prepared reaction solution containing ascorbic acid 2 % (w/v), $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.5 % (w/v) and H_2SO_4 0.6 M. Concentration in PO_4 was then assayed by measuring absorbance at 750 nm with a spectrophotometer and comparison with a KH_2PO_4 standard curve.

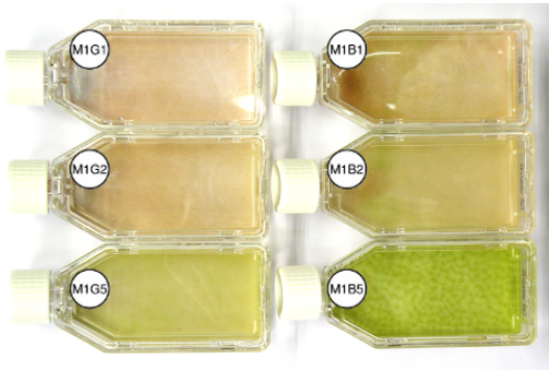

Figure 1: 9-day-old cultures of a screening experiment Representative picture showing 6 of the 18 cultures of a screening experiment. On the left-hand side, from the top to the bottom, cultures M1G1, M1G2 and M1G5 and, on the right-hand side, M1B1, M1B2 and M1B5 (see Table 2 for details). The reddening observed from M1G5 to M1G1 and M1B5 to M1B1 is a typical illustration of the developmental shift from the vegetative stage to the resting and astaxanthin accumulating stage. The M1B5 culture shows the distinguishing reticulated-pattern of motile cells grouping together.

Results

Screening for a medium formulation leading to high vegetative cell density A screening for an optimized *Haematococcus pluvialis* medium was carried out using a blind approach: 18 media were prepared by mixing the 3 components of the Floraseries hydroponic fertilizer (GHE, Fleurance, France) following the experimental design of Table 2. A pre-culture in modified Bold Basal medium (BBM3N) was used to inoculate 10 mL of each culture media at 2 x 10^4 cells mL^{-1}. Cells were grown in continuous light at 30 μmol m^{-2} s^{-1} during 9 days.

Maximum cell densities are presented in Table 2: among the 18 screened media, a maximum cell density of 1 x 10^6 cells mL^{-1}, corresponding to a mean growth rate of 0.41 day^{-1}, was recorded in M1B5 medium (i.e. 1 μL mL^{-1} FloraMicro and 5 μL mL^{-1} FloraBloom) on day 9 while for all other media, except M5B5, the maximum cell density was reached earlier and was 2 to 10-fold lower. M1B5 was the only medium allowing to keep the cells in the green and motile stage after day 9 (Fig. 1) and to sustain growth during 14 days, up to a cell density of 1.73 x 10^6 cells mL^{-1} (data not shown).

One specificity of M1B5 medium, and other 'B5 media, is to have a very high content in phosphate (P), magnesium (Mg) and sulfur (S) which are specifically provided at a high level by the B component (Tables 1 and 2). As for potassium (K), it is provided at highest level in the media prepared with the G component and was thus not supposed to contribute in M1B5 efficiency. Since the B component does not contain a nitrogen (N) source, an-

Table 2: Maximal cell densities obtained in the 18 media Maximal cell densities (mean ± standard deviation, x 10^5 cells mL^{-1}, n=16) measured in a 9-day screening experiment of 18 media obtained by mixing concentrated FloraSeries stock solutions.

		G			B		
	$\mu L.mL^{-1}$	1	2	5	1	2	5
M	1	1.9 ± 0.6	1.4 ± 0.5	1.4 ± 0.5	1.6 ± 0.6	1.6 ± 0.7	**10.0 ± 1.6**
	2	1.2 ± 0.6	1.2 ± 0.5	5.0 ± 1.0	1.9 ± 0.8	5.2 ± 1.5	5.4 ± 1.4
	5	2.3 ± 0.3	2.9 ± 0.7	5.3 ± 1.1	4.2 ± 0.8	3.5 ± 0.9	6.9 ± 1.4

other feature of M1B5 is to have a basal content in N. We thus evaluated which of these 4 nutrients (P, Mg, S and N) could account, alone or interacting with others, for the high macrozoid density obtained in M1B5. Starting from the BBM3N formulation, we decreased the N content by a factor 3, increased P content by a factor 3, and the Mg and S content (as MgSO4) by a factor 5 or 10. Twelve media were generated and used for the culture of *H. pluvialis* in the same experimental conditions as for the previous screening experiment. As shown in Fig. 2a, the highest cell density at day 6 was measured in the media in which P was increased 3 times, other nutrients remaining at their basal BBM3N level. Increasing Mg/S supply was found to have a negative impact on cell growth, independently of N and P supply, while increasing N concentration did not stimulate vegetative cell production and even, in some experiments (data not shown), counterbalance the positive effect of high P supply.

This observation suggested that N and P are interacting in controlling production of vegetative cells. When modifying BBM3N formulation to mimic N and P content of M1B5 (M1B5-like), we indeed observed that it was sufficient to prevent precocious encystment and to stimulate green cells production (Fig. 2b). Moreover, when plotting the 18 screened media on a graph as a function of their initial NO_3 and PO_4 content, the best performing medium, M1B5, appeared as the one with the lowest N:P ratio (± 0.6, Fig. 3). We further observed, by analyzing nitrate and phosphate contents of the media during growth, that there was a very strong relationship between the phosphate consumption by the algae and initial phosphate content of the medium (Fig. 4a). This correlation was not observed for nitrate (Fig. 4b) for which measured depletions in media were low and not related to the initial content. Note that ammonium was not assayed but could act as an N-source at the beginning of the culture.

Evaluation of nutrient limitations in screened media by chlorophyll *a* fluorescence analyzes The role of phosphate as a growth-limiting factor was further evaluated by a non-invasive method based on chlorophyll *a* fluorescence measurement. It is known that, if a nutrient such as phosphate, nitrate or ammonium is added to algal culture limited in that particular nutrient, a change in fluorescence is observable within minutes. This phenomenon has been referred to as "nutrient-induced fluorescence transients" (NIFT) (Beardall et al., 2001) and proved useful to detect true, in situ, nutrient limitations (Holland et al., 2004). In a preliminary experiment, we checked for NIFT occurrence in a 3-week old culture of *H. pluvialis* in BBM3N and observed phosphate-induced NIFT but no nitrate-induced NIFT (Fig. 5a).

The chlorophyll *a* fluorescence analysis at day 6 and day 9 in all 18 screened media revealed that NIFT were induced by phosphate addition in almost all media at day 6 and/or day 9 (data not shown). Conversely, as observed in the preliminary experiment, no NIFT were induced by nitrate addition. When results were analyzed independently of media and age but rather plotted against either relative phosphate uptake or remaining phosphate concentration in medium, the NIFT were observed to occur preferentially when phosphate uptake was greater than 25% of initial content (Fig. 5b).

Evaluation of growth in selected medium M1B5 and in two other media To the best of our knowledge, high-phosphate containing media, such as M1B5, were never used for growing *H. pluvialis*. To evaluate the benefit of such a media on the growth of *H. pluvialis*, we compared growth, in terms of cell density, in M1B5 and two other common media, BBM3N and OHM, containing respectively about 2.5 and 25 times less phosphate than M1B5 and whose NO_3/PO_4 ratio are 4.6 and 23.9, respectively (Fig. 3). Cultures were initiated in 100 ml fresh media at a density of 5 x 10^4 cells mL^{-1} and were run at 30 μmol m^{-2} s^{-1} (low light) and 150 μmol m^{-2} s^{-1} (high light). We observed that, in both BBM3N and OHM, the cells shifted rapidly (around days 4-6) to the palmelloid stage while, in M1B5, they remained in the macrozoid form during the whole experimental period. The developmental shift observed in BBM3N and OHM occurred at both light intensities and coincided with a signif-

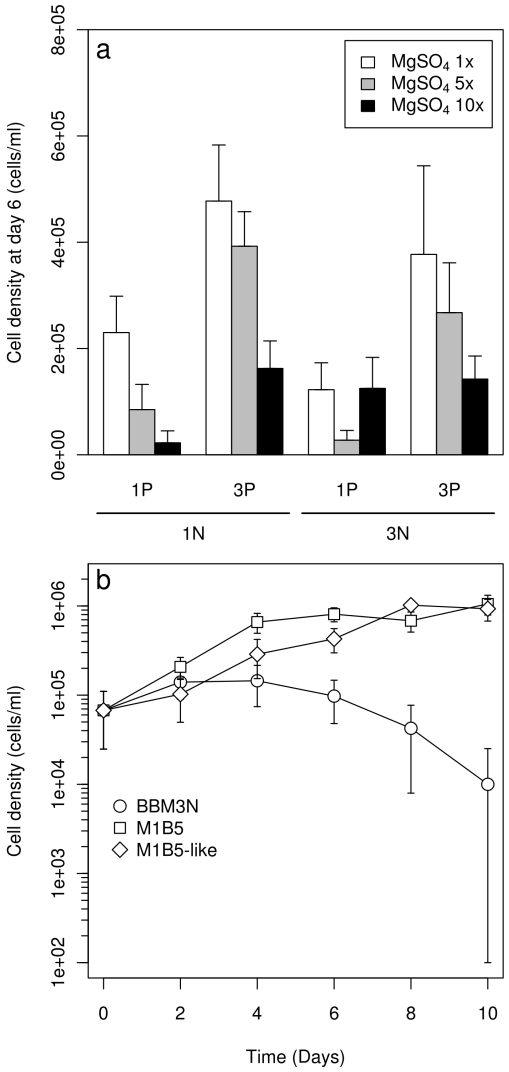

Figure 2: Growth of *H. pluvialis* in modified BBM3N-based media (a) *H. pluvialis* was grown in BBM3N medium modified to contain 3 fold less nitrate (2.9 mM vs 8.8 mM), 3 fold more phosphate (5.1 mM vs 1.7 mM), 5 or 10 fold more $MgSO_4$ (1.5 and 3 mM vs 0.3 mM), either separately or in combination. (b) Starting from the BBM3N formulation, NO_3, NH_4 and PO_4 concentrations (respectively 8.7 mM, 0 mM and 1.7 mM in BBM3N) were modified to mimic M1B5. The resulting medium M1B5-like contained 2.7 mM NO_3, 0.7 mM NH_4 and 4.6 mM PO_4. The observed decrease of cell density in BBM3N is due to adsorption of cysts to walls of flask. These figures are representative results of 3 independent experiments.

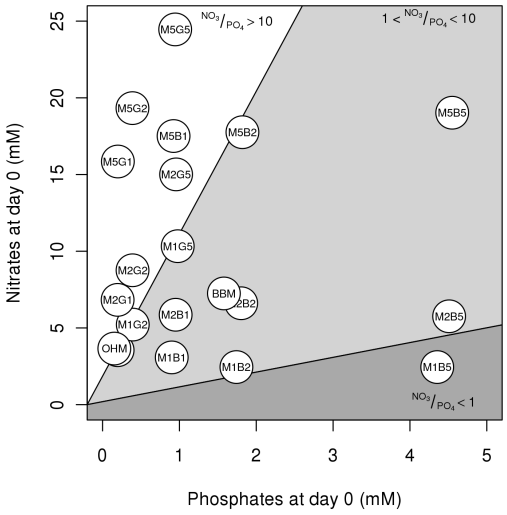

Figure 3: Nitrate-to-phosphate content and relative N:P ratio of the 18 screened media 2D-graph of the initial measured nitrate (y-axis) and phosphate (x-axis) content of 18 screened media. The 2 other common media (OHM and BBM3N) used in this study are also plotted. The plot areas where N:P ratio are below 1, between 1 and 10, and above 10 are filled with dark-grey, light-grey and white, respectively.

icant increase in the carotenoid-to-chlorophyll ratio above 0.5 which is a maximum for *H. pluvialis* green cells (this study, see also Solovchenko et al. (2011)). These observations correlated well with growth measurements: as shown on Fig. 6, a significant increase in cell density was only observed in M1B5 medium. A maximum of 1.4 and 2.1 x 10^6 cells mL^{-1} was measured after 14 days at low and high light, respectively.

Effect of N & P deficiencies on M1B5 cultures The above observations tend to show that the M1B5 medium has specific impacts on growth and development of *H. pluvialis* by stimulating cell division and preventing precocious cysts formation. Nutrient uptake measurements and NIFT assays indicated that the high-phosphate content and/or the low NO_3/PO_4 ratio of M1B5 could be at the origin of these responses. To further test this hypothesis, we transferred M1B5 exponentially growing cells to media containing lower phosphate (high N:P ratio) or lower nitrate (low N:P ratio) and evaluated the quantitative (cell density) and developmental (cell types, carotenoid-to-chlorophyll ratio) consequences of these treatments.

Independently of light intensity, transfer to BBM3N or phosphate-deprived BBM3N led to a dramatic decrease in cell densities due to induced cell death as observed under the microscope (Fig. 7a & b). In high light, cell death occurred rapidly and was almost complete after 2 days while, in low light, the surviving cells shifted to the palmelloid stage: 95% of them were palmella at the end of the experiment. Conversely, when cells are transferred to fresh M1B5 or nitrate-deprived BBM3N, cell density still increased. Furthermore, more than 90% of the cells stayed in motile macrozoid stage at either low or high light. Strikingly, the maximum cell density was observed in the nitrate-deprived BBM3N media in high light. In these conditions, cells accumulated carotenoids, they became red and carotenoid-to-chlorophyll ratio increased up to 0.8,

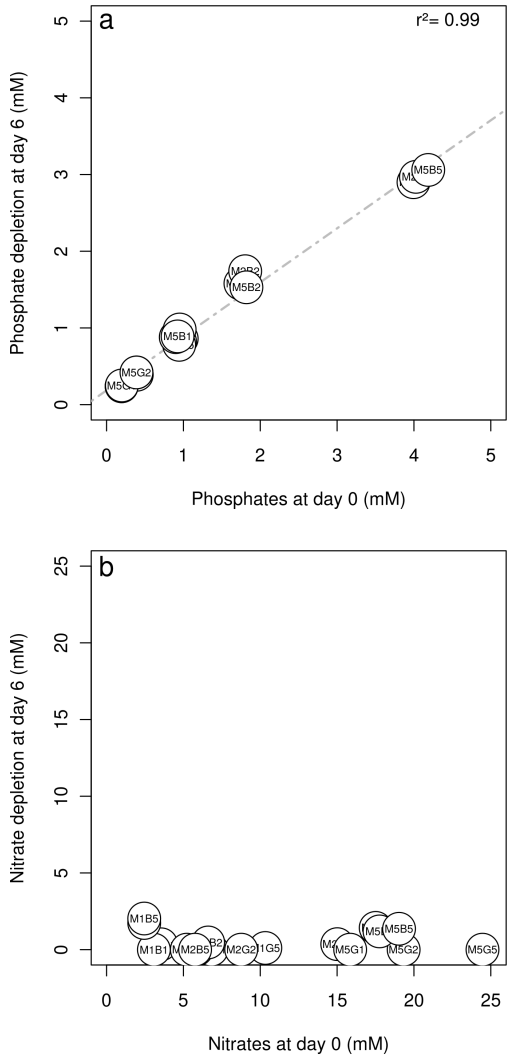

Figure 4: Phosphate and nitrate uptake at day 6 in relation with initial content Phosphate and nitrate concentrations in culture media were measured in fresh media (initial content) and at day 6. Phosphate and nitrate depletion were respectively plotted against initial phosphate content (a) and initial nitrate content (b) of screened media.

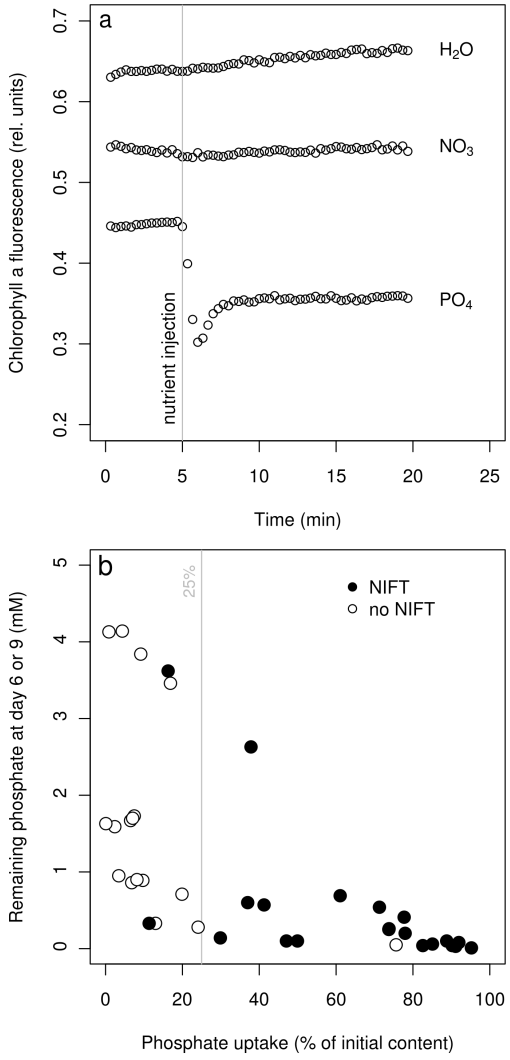

Figure 5: NIFT occurrence at day 6 or day 9 in screened media in relation with nutrient uptake (a) Representative result of a preliminary experiment where NIFT occurrence was analyzed in 3-week old BBM3N stock culture following addition of either 20 μL H_2O, 500 μM $NaNO_3$ (NO_3) or 500 μM K_2HPO_4 (PO_4). (b) Phosphate-induced NIFTs plotted as 2-D graph of actual phosphate content vs phosphate uptake. Empty dots: no NIFT observed. Filled dots: phosphate-induced NIFT.

while remaining at the macrozoid stage (Fig. 7b). In this specific case, carotenoids accumulation did not occur simultaneously with growth arrest as it is the case in the BBM3N and phosphate-deprived media at high light.

Discussion

In this study, medium optimization was performed by screening three-component hydroponic fertilizer based media. This strategy allowed us to evaluate, on a limited number of media (18), the effect of a large range of individual nutrient supplies together with a large range of macro-nutrients ratios. For example, nitrate and phosphate ranges were 2.20-28.85 mM and 0.18-4.33 mM, respectively, while their relative N:P ratio ranged from 0.6 to 111.8. With this experimental set-up, the relative supply of micro-nutrients was not allowed to be optimized since micro-elements are provided by only one of the three fertilizer components. However, according to the work of Fábregas et al. (2000) micro-elements do not contribute much in optimization and even their omission has been shown to have a limited growth-inhibitory impact on *H. pluvialis* (Domínguez-Bocanegra et al., 2004; Hagen et al., 2001).

Our screening clearly led to the identification

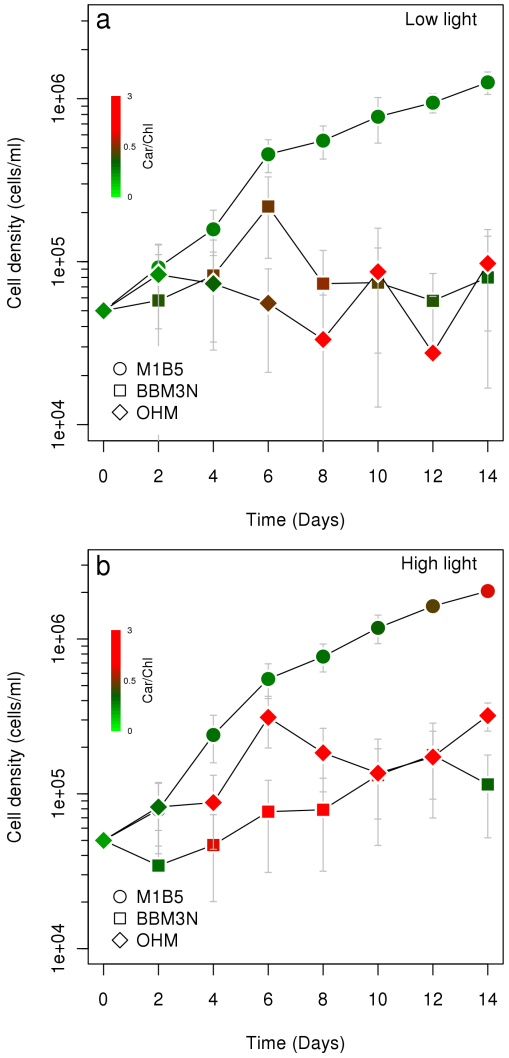

Figure 6: Growth of *H. pluvialis* in M1B5, BBM3N and OHM media at two light intensities Cell densities, chlorophyll and carotenoids content were measured at 2 days interval during a 14 days culture in M1B5, BBM3N and OHM media either at 30 μmol m^{-2} s^{-1} (a) or 150 μmol m^{-2} s^{-1}. The carotenoid-to-chlorophyll ratio of each sample is represented on a color-coded scale from light-green (0) to red (3). A non-linear scale was used to visualize "green" stage (car:chl < 0.5) as green. This figure is a representative result of two independent experiments. Data are means ± standard deviations (n=8). The fluctuating cell densities measured in BBM3N and OHM from day 6 are due to partial adsorption of cyst to walls of flask and production of macrozoids from cyst (concomitant to a drop in Car/Chl ratio).

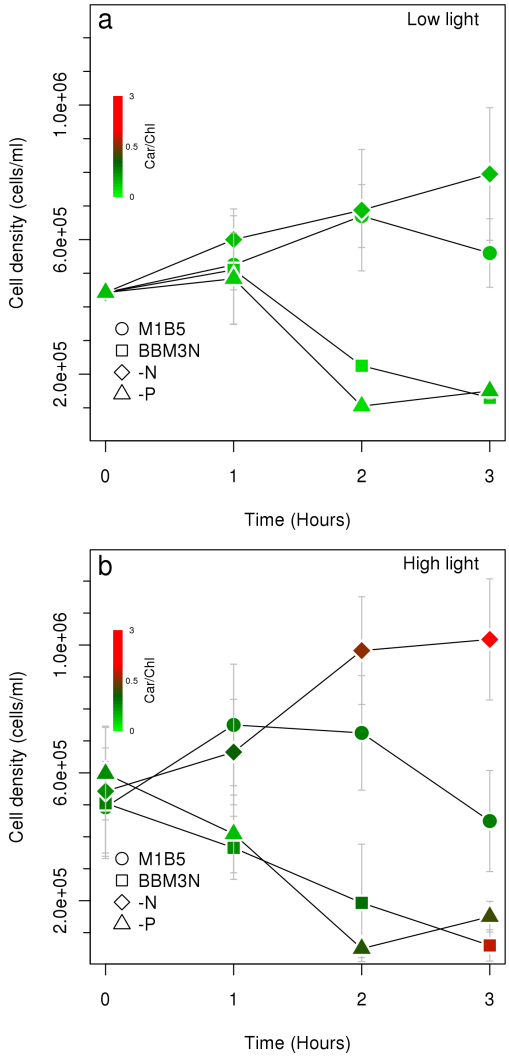

Figure 7: Impact of nitrate or phosphate starvation on the growth of *H. pluvialis* Cell densities, chlorophyll and carotenoid contents were measured at 24-hours interval during 3 days following the transfer of M1B5 growing cells to Bold Basal medium (BBM3N), N-depleted BBM3N (-N) and P-depleted BBM3N (-P) at either at 30 μmol m^{-2} s^{-1} (a) or 150 μmol m^{-2} s^{-1} (b). The carotenoid-to-chlorophyll ratio of each sample is represented on a color-coded scale from light-green (0) to red (3). A non-linear scale was used to visualize "green" stage (car:chl < 0.5) as green. This figure is a representative result of 2 independent experiments. Data are means ± standard deviations (n=8).

of one optimal formulation, which is composed of 1 μL ml^{-1} FloraMicro and 5 μL ml^{-1} FloraBloom (see Materials & Methods for composition) and was labeled "M1B5". During 9 days screening experiments, growth was indeed sustained until day 9 in only 2 media (M1B5 and M5B5, data not shown), but cell density was the highest in M1B5 (1 x 10^6 cells mL^{-1}, Table 2). When we compared growth in M1B5 with BBM3N and OHM, we observed that vegetative growth was sustained at least during 2 weeks and a cell density of 2 x 10^6 cells mL^{-1} was reached under high light intensity. These results represent a minimum 5-fold improvement compared with previous studies in similar batch-mode conditions (Borowitzka et al., 1991; Cifuentes et al., 2003; Domínguez-Bocanegra et al., 2004; Kaewpintong et al., 2007) and with the maximal cell densities of 1.8 and 3.2 x 10^6 cells mL^{-1} obtained in this study with BBM3N and OHM, respectively. For BBM3N, recorded maximal cell density was in the range of published data obtained in similar growing condi-

tions (Harker et al., 1996; Tripathi et al., 1999; Domínguez-Bocanegra et al., 2004), while for OHM, it correspond to steady-state density obtained by Fábregas et al. (2000) in a semi-continuous mode with daily addition of fresh medium. The efficiency of M1B5 can not be explained by absolute availability of nutrients since, in our screening, other formulations contained as much nutrients, like nitrate or phosphate, or even more. Rather, we observed that M1B5 had a specific physiological effect by delaying the shift to encystment. This effect was neither observed for any of the other screened media, nor for BBM3N or OHM, where a maximum in cell density observed after 3 or 6 days preceded a developmental shift to the red resting stage.

Although phosphate is not the only nutrient that is at high level in M1B5, our results indicate that high phosphate is the key feature leading to its beneficial effect on cell growth. Firstly, we showed that among nutrients that are present in higher amount in M1B5, phosphate accounted, alone, for the increase in cell density. Secondly, we observed that phosphate uptake increased with available phosphate in medium and, finally, we also demonstrated, using the NIFT approach, that cells are rapidly behaving as phosphate-limited whatever the initial phosphate content of the medium. Compared to nitrate, phosphate has received less attention in optimization approaches and has long been considered to promote growth at moderate or low concentration (± 0.5 mM, Borowitzka et al. (1991); Fábregas et al. (2000)) while it could promote carotenogenesis at higher concentration (up to 0.9 mM, Borowitzka et al. (1991)). However, in other reports, carotenoid accumulation has been shown to be reduced when phosphate supply was increased above 0.85 mM (up to 3.4 mM, Harker et al. (1996)). Harker et al. (1996) even reported a positive impact of a high phosphate concentration (>3 mM) on the growth of *H. pluvialis*.

In addition to have a high phosphate content, M1B5 also is remarkable for having the lowest nitrate-to-phosphate ratio (0.6) among the 18 screened media. Both features are rarely found in already known *H. pluvialis* media: most of them have a moderate phosphate content (± 0.2-2 mM) and a N:P ratio largely in favor of N (± 7-20) (Domínguez-Bocanegra et al., 2004; Fábregas et al., 2000). There are only a few reports on *H. pluvialis* growing in N:P ratio close or below one but, in all cases that we identified, the low N:P conditions were beneficial to growth (Dalay et al., 2007; Harker et al., 1996; Hagen et al., 2001). In the present study, we showed that the transfer from the M1B5 medium to low-phosphate containing BBM3N medium led to a rapid cell death while it was not the case if nitrate was omitted in media. In this case where N:P ratio was null, growth was not inhibited during the time-course of the experiment and the cells stayed at the vegetative motile stage and accumulated carotenoids under increased irradiance without shifting to resting stage as it was already observed in similar conditions by Hagen et al. (2001). Moreover, we observed that increasing N-supply together with P-supply reduced the positive effect of high P on macrozoids production. The low N:P ratio thus appeared to be a critical parameter that maintains actively dividing cells at the macrozoid stage.

In most organisms, phosphate accumulates inside the cell as a linear polymer forming intracellular polyphosphate granules (PolyP) (Kulaev and Kulakovskaya, 2000). In a preliminary experiment, we looked for the presence of PolyP granules in *H. pluvialis* by microscopy with the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI), following the method by Tijssen et al. (1982) and we observed that granules were visible in M1B5 growing macrozoids (data not shown). The function of PolyP, which has been essentially studied in procaryotes, are numerous, including stress response, quorum sensing, motility, etc... (for review, see Rao et al.). In microalgae, roles in osmotic stress response, in heavy metal tolerance or phosphate storage have been already proposed but the identification of more functions will undoubtedly occur as the interest for PolyP in eucaryote increases. In this context, the relation between phosphate-supply, PolyP accumulation and cell cycle control in *H. pluvialis* is of particular interest and requires deeper investigations.

In conclusion, by a medium screening strategy based on a 3-component commercial fertilizer, we obtained a new medium formulation that proved to be efficient, at least in batch culture, in promoting high density culture of *H. pluvialis* macrozoids and in post-poning the development shift to the resting stage. We demonstrated that these effects were a response to both the unusual phosphate content and the below-one N:P ratio of this medium. Even if the exact impact of these parameters on the physiology of *H. pluvialis* remains to be determined, achievement of high vegetative cell density culture in this easy to prepare and low-cost media could be evaluated for the production of astaxanthin in either 1- or 2-step strategies.

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