Comparison of the accumulation of astaxanthin in *Haematococcus pluvialis* and other green microalgae under *N*-starvation and high light conditions

M. Orosa, J.F. Valero, C. Herrero & J. Abalde[1]

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Abstract

Haematococcus pluvialis gave the highest astaxanthin accumulation rate (2.7 mg l^{-1} day^{-1}) and total astaxanthin content (22.7 mg g^{-1} biomass). Astaxanthin accumulation in *Neochloris wimmeri*, *Protosiphon botryoides*, *Scotiellopsis oocystiformis*, *Chorella zofingiensis* and *Scenedesmus vacuolatus* was, respectively, 19.2, 14.3, 10.9, 6.8 and 2.7 mg astaxanthin g^{-1} biomass, respectively.

Introduction

Primary carotenoids are those synthesized under normal, favourable growth conditions. So-called secondary carotenoids are produced under unfavourable nutritional conditions, especially nitrogen deficiency (Britton 1988). Probably the most well-known and studied secondary carotenoid is astaxanthin (3,3'-dihydroxy-*β*-*β*-carotene-4,4'-dione) which is commonly used in aquaculture as the main source of the red flesh of salmonid fishes. Moreover, it is highly effective as a quencher of singlet oxygen, making it an important antioxidant for the pharmaceutical and cosmetic industries (Munzel 1981).

In recent years, the search for natural sources for the production of the ketocarotenoid astaxanthin has been mainly focused on the green microalga *Haematococcus pluvialis.* However, *Haematococcus* is not the only microalga able to accumulate significant amounts of

[1] Laboratorio de Microbioloxía, Departamento de Bioloxí Celular e Molecular, Universidade da Coruña, Rúa Alejandro de la Sota no, 15008, A Coruña, Spain
Author for correspondence (Fax: +981 167065; E-mail: abaldej@udc.es)

astaxanthin and other related ketocarotenoids. The capability to synthesize secondary carotenoids under environmental stresses is widely spread over the green microalgae, probably as a defense mechanism against environmental injuries.

Secondary carotenoid formation can be influenced by nitrogen-limitation, strong light intensity, pH, salinity and some organic nutrients such as acetate; in general, almost any factor which causes growth cessation can have an effect on secondary carotenoid accumulation (Boussiba & Vonshak 1991, Harker *et al.* 1996, Kobayashi *et al.* 1997, Fábregas *et al.* 1998, Boussiba 2000, Orosa *et al.* 2001). However, more knowledge of the triggering factors leading to a high secondary carotenoid accumulation in different microalgae is required to develop strategies towards optimization of astaxanthin production in large-scale algal cultures.

Several green microalgae have been tested as potential sources of keto-carotenoids (Arad *et al.* 1993, Rise *et al.* 1994, Gouveia *et al.* 1996, Zhang & Lee 1999, Hanagata& Dubinsky 1999, Hagen *et al.* 2000), however, the resulting data are not comparable due to the different assayed conditions.

The aim of this work was to study secondary carotenoid accumulation during culture of several green microalgae under identical inductive secondary carotenoid conditions in order to compare and maximize astaxanthin accumulation.

Materials and methods

The microalgae *Haematococcus pluvialis* CCAP-34/7 and *Neochloris wimmeri* CCAP-213/4 were obtained from the Culture Collection of Algae and Protozoa of the Windermere Laboratory, Cumbria, UK. *Scenedesmus vacuolatus* SAG-211/15, *Scotiellopsis oocystiformis* SAG-277/1, *Chlorella zofingiensis* SAG-211/14 and *Protosiphon botryoides* SAG-731/1a were obtained from the Culture Collection of the University of Göttingen, Germany.

The strains were cultured in modified Bold's Basal Medium with 0.25 g l^{-1} of NaNO3 (MBBM) as described by Orosa *et al.* (2000) to obtain enough biomass for experiments. Green cells in the mid-growth phase were harvested by centrifugation at 1000 *g* for 5 min and resuspended in different culture media to induce secondary carotenoid accumulation. Cultures in modified Bold's Basal Medium (Orosa *et al.* 2000) without $NaNO_3$ (MBBM-N) were used as control cultures. The secondary carotenoid inductive conditions used were: (a) initial acid pH (MBBM-N adjusted to pH 4), (b) salinity stress (NaCl 300 mM or 600 mM), (c) mixotrophy (MBBM-N with sodium acetate 100 mM). All these experiments were carried out under strong light intensity (350 μmol photon m^{-2} s^{-1}). The initial cell concentration was adjusted to 500 mg l^{-1} in all the tested conditions for each microalga. All cultures were carried out in triplicate. Results are always expressed as mean ± standard deviation.

Biomass determination and pigment analysis (spectrophotometrically and by HPLC) were performed as described by Orosa *et al.* (2000).

Astaxanthin accumulation rates were calculated as follows:

$$\text{Astaxanthin accumulation rate} = [\text{astaxanthin}]_t - [\text{astaxanthin}]_0/t,$$

where $[\text{astaxanthin}]_t$ is the concentration of astaxanthin (mg l^{-1}) corresponding to day t , $[\text{astaxanthin}]_0$ is the concentration of astaxanthin (mg l^{-1}) corresponding to day 0 of culture, and t is time in days.

Maximum astaxanthin accumulation rate (MAR), was defined as the highest rate obtained for each tested condition with each microalga. Average astaxanthin accumulation rate (AAR) was defined as the value of the astaxanthin accumulation rate corresponding to $t = 24$.

For each microalga, data of maximum astaxanthin accumulation rate corresponding to each assayed condition were statistically analyzed by one-way analysis of variance (ANOVA), followed by a Duncan post hoc comparison (SPSS 10.0). The same statistical analysis was employed to compare data from the different microalgae, using the highest maximum rate obtained in each microalga. Differences were considered significant when $p < 0.05$.

Results

In all the microalgae studied, when green cells were harvested in the mid-growth phase of growth and transferred to nitrate-free medium, exposing them to strong light and other secondary carotenoid inductive conditions (salinity stress, acid initial pH and acetate), they became red-orange and synthesized a thick cell wall accompanied by a cell size increase.

Maximum (MAR) and average (AAR) accumulation rates of astaxanthin and the day these values were obtained of the six tested strains grown under different secondary carotenoid inductive conditions are summarized in Table 1.

Table 2 shows maximum total astaxanthin content in biomass (mg g^{-1} biomass), and the percentage of astaxanthin with respect to total carotenoid on the day of highest total astaxanthin content in biomass.

HPLC analysis indicated that astaxanthin (mainly in esterified form) was the main pigment in all the microalgae studied (Table 2). Besides astaxanthin, other ketocarotenoids such as cantaxanthin, echinenone, adonixanthin and adonirubin were also detected, although at lower concentrations (Figure 1).

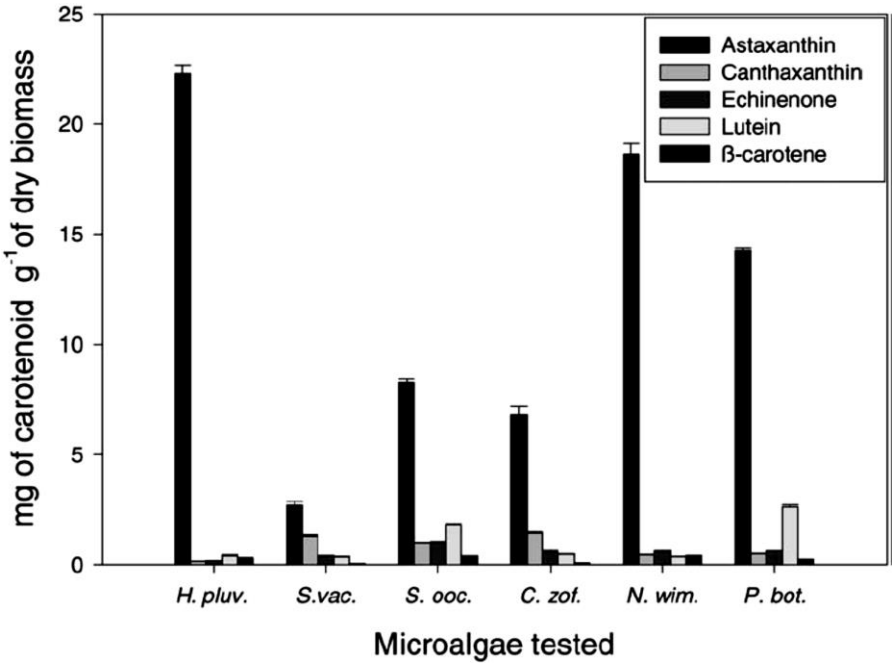

Fig. 1. Concentration of total astaxanthin, canthaxanthin, echinenone, lutein and *β*-carotene accumulated in all the microalgae tested under the conditions that induce the highest MAR corresponding to the last day of culture (24th day): *Haematoccocus pluvialis* (100 mM of sodium acetate); *Scenedesmus vacuolatus* (NaCl 300 mM); *Scotiellopsis oocystiformis* (100 mM of sodium acetate); *Chlorella zofingiensis* (NaCl 300 mM); *Neochloris wimmeri* (NaCl 300 mM); *Protosiphon botryoides* (NaCl 300 mM).

S. vacuolatus synthesized canthaxanthin in a relatively high concentration, reaching maximum values of 1.3 ± 0.1 mg g^{-1}, and although the carotenoid astaxanthin appeared in free as well as in esterified form, it only accounted for 40–50% of the total carotenoids accumulated in this microalga (Table 1).

Haematococcus pluvialis

Salinity stress caused a rapid increase in cell mortality in cultures of *Haematococcus*. The cell density was very low beyond the third day, corresponding with the lowest MAR and AAR (Table 1) and lowest concentration in biomass (Table 2).

The statistical analysis showed that the optimum conditions to obtain MAR of astaxanthin were in cultures containing sodium acetate or with an initial acid pH (Table 1). The period of maximal accumulation was between 3 and 12 days, depending on the condition assayed (Table 1), and astaxanthin was mainly detected in free or monoester form (Figure 2). Once this period of rapid astaxanthin synthesis was finished, the concentration of this carotenoid continued increasing, especially as diesters of astaxanthin (Figure 2), but at lower rate.

Astaxanthin concentration in biomass reached a maximum of 22.2 mg g−1 on the 12th day, and beyond this day, suffered little variations (Table 2).

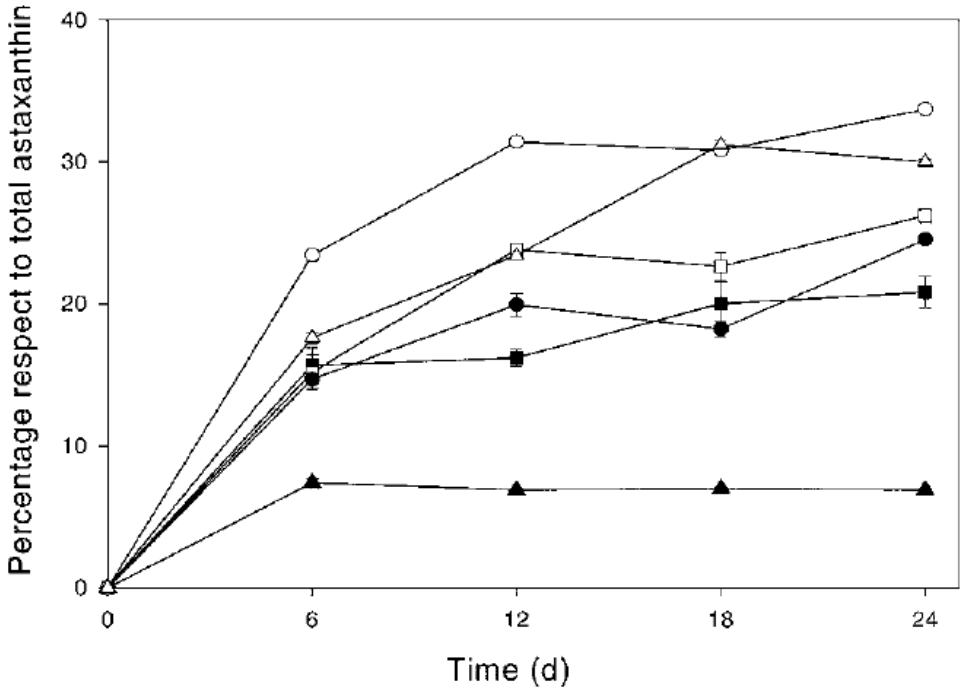

Fig. 2. Percentage of astaxanthin diesters with respect to total astaxanthin along the time, under the conditions that induce maximum astaxanthin accumulation rates (MAR) for each studied microalga. ■, *Haematococcus pluvialis* (100 mM of sodium acetate), □ *Scenedesmus vacuolatus* (NaCl 300 mM), ● *Scotiellopsis oocystiformis* (100 mM of sodium acetate), ○ *Chlorella zofingiensis* (NaCl 300 mM), ▲ *Neochloris wimmeri* (NaCl 300 mM), ⌂ *Protosiphon botryoides* (NaCl 300 mM). Bars indicate standard error.

Scenedesmus vacuolatus

Statistical analysis showed no significant differences in the MAR in all the assayed conditions in *S. vacuolatus*. In addition, the amount of astaxanthin accumulated in biomass did not exceed 3 mg g^{-1}. This concentration was clearly lower than that obtained in *Haematococcus*.

An important feature about *S. vacuolatus* secondary carotenoid accumulation was the relative high concentration of cantaxanthin, reaching maximum values of 1.3 ± 0.1 mg g^{-1} (Figure 1).

Scotiellopsis oocystiformis

The presence of acetate or a NaCl in the highest concentration tested (600 mM) were the factors that more strongly affected the MAR in *S. oocystiformis*. The lower NaCl concentration assayed (300 mM) was the next influential factor, followed by an initial acid pH (Table 1).

The MAR was lowest in control cultures without other inductive conditions other than N-starvation and strong light. Although maximal astaxanthin concentration was higher than in *S. vacuolatus*, the maximum value 10.9 mg g^{-1} was barely half the concentration of that in *Haematococcus* (Table 2).

Table 1. Maximum astaxanthin accumulation rates (MAR) with the period in days (*t*) used to calculate them, and average astaxanthin accumulation rates (AAR) corresponding to all the microalgae studied under all the tested conditions.

	MAR ($mg\ l^{-1}\ day^{-1}$)	*t* (days)	AAR ($mg\ l^{-1}\ day^{-1}$)
Haematococcus pluvialis			
Control	2.4 ± 0.1	12	1.7 ± 0.1
pH 4.0	2.6 ± 0.0	6	2.0 ± 0.1
NaCl 300 mM	0.5 ± 0.0	3	0.5 ± 0.0
NaCl 600 mM	0.3 ± 0.1	6	0.3 ± 0.1
Sodium acetate	2.7 ± 0.1	3	1.5 ± 0.0
Scenedesmus vacuolatus			
Control	0.1 ± 0.0	6	0.1 ± 0.0
pH 4.0	0.1 ± 0.0	3	0.1 ± 0.0
NaCl 300 mM	0.2 ± 0.0	6	0.1 ± 0.0
NaCl 600 mM	0.2 ± 0.0	3	0.1 ± 0.0
Sodium acetate	0.2 ± 0.0	3	0.1 ± 0.0
Scotiellopsis oocystiformis			
Control	0.5 ± 0.0	6	0.4 ± 0.0
pH 4.0	0.6 ± 0.0	3	0.4 ± 0.0
NaCl 300 mM	0.8 ± 0.0	18	0.6 ± 0.0
NaCl 600 mM	0.9 ± 0.0	12	0.6 ± 0.0
Sodium acetate	1.0 ± 0.0	6	0.6 ± 0.0
Chlorella zofingiensis			
Control	0.5 ± 0.1	6	0.3 ± 0.0
pH 4.0	0.4 ± 0.0	3	0.2 ± 0.0
NaCl 300 mM	0.8 ± 0.1	6	0.3 ± 0.0
NaCl 600 mM	0.3 ± 0.0	3	0.1 ± 0.1
Sodium acetate	0.4 ± 0.1	3	0.3 ± 0.0
Neochloris wimmeri			
Control	1.4 ± 0.0	18	1.2 ± 0.0
pH 4.0	1.2 ± 0.0	18	1.1 ± 0.0
NaCl 300 mM	2.2 ± 0.0	6	1.4 ± 0.0
NaCl 600 mM	0.3 ± 0.0	3	0.3 ± 0.0
Sodium acetate	1.0 ± 0.2	3	0.6 ± 0.0
Protosiphon botryoides			
Control	1.0 ± 0.1	12	0.7 ± 0.0
pH 4.0	0.9 ± 0.1	12	0.6 ± 0.0
NaCl 300 mM	1.2 ± 0.2	6	0.7 ± 0.0
NaCl 600 mM	0.7 ± 0.1	6	0.4 ± 0.0
Sodium acetate	1.2 ± 0.2	6	0.6 ± 0.0

Chlorella zofingiensis

According to the statistical analysis a concentration of 300 mM of NaCl in the culture medium had the greatest effect on the MAR in *C. zofingiensis* (Table 1). Other conditions resulted in a lower value, without significant differences between all of them.

Astaxanthin concentration in *C. zofingiensis* reached maximum values of 6.8 $mg\ g^{-1}$ at the lower concentration of NaCl, almost twice the quantity observed in cultures with the highest concentration of NaCl (600 mM) (Table 2), due to the high cell mortality under stringent salinity.

Neochloris wimmeri

Neochloris wimmeri had the special ability of synthesizing small concentrations of astaxanthin (mainly in free-form) during vegetative growth. *N. wimmeri*, just like *C. zofingiensis*, showed the highest MAR in cultures with a concentration of 300 mM of NaCl, also resulting in a relatively high rate of cell mortality in the highest NaCl concentration assayed (600 mM) (Tables 1 and 2). Astaxanthin formation reached more than 19 mg g^{-1}, which was almost threefold higher than in cultures of *S. oocystiformis* and *C. zofingiensis* and it was slightly lower than in cultures of *Haematococcus* (Table 2).

Table 2. Maximum astaxanthin concentration in biomass (mg astaxanthin g^{-1} of dry weight) reached by the microalgae studied under each tested condition and the day of maximal accumulation, and percentage of astaxanthin with respect to the total carotenoids accumulated.

	Astaxanthin (mg g−1)	Day	Percentage of astaxanthin
Haematococcus pluvialis			
Control	21.8 ± 0.4	12	87.1 ± 0.8
pH 4.0	22.7 ± 0.4	18	88.5 ± 0.6
NaCl 300 mM	8.1 ± 0.4	3	50.4 ± 0.2
NaCl 600 mM	5.8 ± 0.9	3	39.8 ± 2.7
Sodium acetate	22.2 ± 0.7	12	92.2 ± 0.5
Scenedesmus vacuolatus			
Control	1.5 ± 0.3	12	42.8 ± 2.5
pH 4.0	1.9 ± 0.0	12	46.9 ± 0.6
NaCl 300 mM	2.7 ± 0.2	24	53.3 ± 1.4
NaCl 600 mM	1.8 ± 0.1	12	40.8 ± 0.7
Sodium acetate	1.7 ± 0.3	6	39.8 ± 1.1
Scotiellopsis oocystiformis			
Control	6.4 ± 0.5	24	53.8 ± 1.9
pH 4.0	7.0 ± 0.5	24	59.3 ± 0.3
NaCl 300 mM	9.0 ± 0.2	24	66.9 ± 0.4
NaCl 600 mM	9.2 ± 0.0	12	63.0 ± 0.3
Sodium acetate	10.9 ± 0.8	6	73.4 ± 1.6
Chlorella zofingiensis			
Control	6.3 ± 0.5	24	65.7 ± 0.9
pH 4.0	5.9 ± 0.0	24	67.4 ± 0.9
NaCl 300 mM	6.8 ± 0.4	24	69.8 ± 0.2
NaCl 600 mM	3.5 ± 0.2	3	63.8 ± 3.4
Sodium acetate	6.5 ± 0.3	24	61.1 ± 1.4
Neochloris wimmeri			
Control	19.3 ± 0.1	24	90.2 ± 0.3
pH 4.0	17.3 ± 0.1	24	87.8 ± 0.1
NaCl 300 mM	18.6 ± 0.5	24	88.5 ± 0.5
NaCl 600 mM	5.1 ± 0.4	3	47.3 ± 0.2
Sodium acetate	12.7 ± 0.3	24	88.0 ± 3.0
Protosiphon botryoides			
Control	14.3 ± 0.9	24	76.2 ± 0.7
pH 4.0	13.2 ± 0.6	24	75.4 ± 1.0
NaCl 300 mM	14.3 ± 0.1	24	75.0 ± 0.3
NaCl 600 mM	13.8 ± 0.9	12	85.6 ± 2.0
Sodium acetate	12.9 ± 0.2	24	70.7 ± 0.3

Protosiphon botryoides

None of the conditions assayed in *P. botryoides* significantly stimulated astaxanthin accumulation with respect to control cultures. Astaxanthin content obtained in cultures of this microalga reached the highest value of 14.3 mg g^{-1}, corresponding to the 24th day of culture (Table 2).

Comparison of the six microalgae studied

The MAR of all microalgae studied, under the conditions that induce maximum astaxanthin accumulation rates (MAR) for each studied microalga (Table 1) were compared, and the statistical analysis showed significant differences between them (Figure 3). The Duncan test distributed them into 5 different categories.

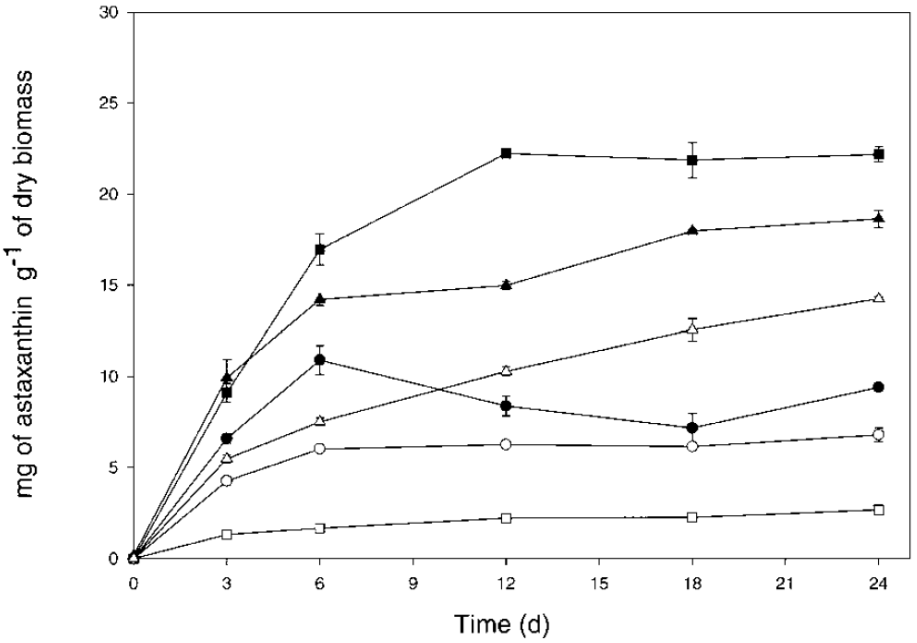

Fig. 3. Accumulation of total astaxanthin (mg of astaxanthin g^{-1} of dry biomass) along the time, under the conditions that induce maximum astaxanthin accumulation rates (MAR) for each studied microalga. ■ *Haematoccocus pluvialis* (100 mM of sodium acetate), □ *Scenedesmus vacuolatus* (NaCl 300 mM), ● *Scotiellopsis oocystiformis* (100 mM of sodium acetate), ○ *Chlorella zofingiensis* (NaCl 300 mM), ▲ *Neochloris wimmeri* (NaCl 300 mM), ⌂ *Protosiphon botryoides* (NaCl 300 mM). Bars indicate standard error.

The first category, having the highest MAR, corresponded to cultures of *Haematococcus* containing sodium acetate 100 mM with an average value of 2.7 ± 0.1 mg l^{-1} day^{-1}.

The second category was composed of *N. wimmeri* cultureswith a concentration of 300mMof NaCl, with a value of 2.2 ± 0.0 mg l^{-1} day^{-1}.

The third category of microalgae determined by the Duncan test corresponded to *P. botryoides* in cultures with the lower assayed concentration of NaCl (300 mM), and *S. oocystiformis* in

cultures with a concentration of sodium acetate of 100 mM, reaching values of 1.2 ± 0.2 and 1.0 ± 0.0 mg l^{-1} day^{-1}, respectively (Table 1).

The fourth category with a lower MAR corresponded to cultures of *C. zofingiensis* with a maximum value of 0.8 ± 0.1 mg l^{-1} day^{-1}.

The last category obtained consisted of *S. vacuolatus* cultures whose maximal astaxanthin accumulation rate was only 0.2 mg l^{-1} day^{-1}.

The duration of the MAR under the best conditions for each microalga was between the 3rd and the 12^{th} day from the transference to secondary carotenoidinductive conditions. However, astaxanthin synthesis continued almost until the last day of culture in all the microalgae studied, except in *Haematococcus* where no more than 12 days were necessary to obtain the highest astaxanthin concentration per gram of dry biomass as seen in Table 2.

Discussion

Pigment analysis by HPLC showed that in all six microalgae studied, nitrate starvation and high light intensity (control conditions) are sufficient to produce ketocarotenoids synthesis and accumulation (Figure 1). The accumulated carotenoids are essentially the same in all chlorophycean algae capable of accumulating astaxanthin as secondary carotenoid (Arad *et al.* 1993, Rise *et al.* 1994, Gouveia *et al.* 1996, Zhang *et al.* 1999, Hanagata& Dubinsky 1999, Hagen *et al.* 2000). However, the relative ratio between the different carotenoids varies with the algae and with the age of the culture as well as with the inductive secondary carotenoid conditions assayed (Figure 2).

In recent scientific literature on secondary carotenoid accumulation in different green microalgae, the ketocarotenoid, echinenone, has not been reported but, according to our data (Orosa *et al.* 2000), this could be due to problems in the analytical techniques used, since its relatively lower concentration and its peak location between the astaxanthin monoesters makes its detection difficult. The same occurs with adonixanthin, a carotenoid found in all the microalgae studied.

In this study, these carotenoids were found in all the microalgae studied, and their relative concentration varied with the aging of the cultures. These results could indicate certain homogeneity in the pathway from *β*-carotene to astaxanthin in all the green microalgae studied, but more work is still necessary to learn more about the ketocarotenoid biosynthesis pathway.

An exceptional fact is the presence of astaxanthin in cultures of *N. wimmeri* during the logarithmic growth phase, although in free-form and at low concentrations. Once transferred to nitrate-free medium, it began a progressive esterification of astaxanthin. The presence of astaxanthin in the logarithmic growth phase could suggest some nutritional deficiency or environmental stress during this growth phase; however, it was not detected.

Haematococcus cultures showed the highest maximum astaxanthin accumulation rate and the highest total astaxanthin content in biomass (mg astaxanthin g^{-1} dry biomass) of all the microalgae studied. The best conditions for obtaining this high ketocarotenoids content are under mixotrophic growth with acetate or under an acid initial pH once transferred to a stronger light and nitrate-free medium. *Haematococcus* also showed a limited resistance to salinity stress (Table 1) and the high cell mortality induced very low astaxanthin concentrations. The other microalgae studied resisted a salinity of at least 300 mM of NaCl and in *N. wimmeri*, *S. oocystiformis* and *C. zofingiensis* cultures, salinity stress induced the highest astaxanthin synthesis. This property together with the additional advantage of their relative fast growth make these microalgae good candidates for successful culture in open ponds (Orosa *et al.* 2000).

The highest MAR occurred between the 3th and the 12th day of culture in all the microalgae; however, the highest astaxanthin concentration occurred later because cells continued synthesizing ketocarotenoids, mainly astaxanthin diesters, for more time, in some cases even until the last day of the experiment (Table 2). These data show the existence of two different periods in the astaxanthin accumulation. The first period corresponds to the 12 early days after the transfer to the inductive conditions characterized by a rapid astaxanthin accumulation in all the algae studied, mainly in free and monoester form (Figure 2). The second period, after the 12th day, is characterized by a slower astaxanthin accumulation, mainly in diester form, and corresponds with the larger cell size and higher astaxanthin content per cell (Figure 3).

In conclusion, the data reported here can be used to optimize the secondary carotenoid inductive conditions and they suggest the possible modification of the carotenoid composition in the microalgae biomass by manipulation of environmental conditions and time of accumulation. The optimization of the secondary carotenoids inductive conditions together with the fast growth and strong resistance to environmental stress conditions of microalgae as *Neochloris wimmeri*, *Protosiphon botryoides* and *Scotiellopsis oocystiformis* could make them a potential alternative to *Haematococcus* and they could be possible candidates for mass production of astaxanthin.

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