Chinese Journal of Oceanology and Limnology
Vol.20, No.4, P.358－364, 2002

DYNAMIC CHANGES OF INORGANIC NITROGEN AND ASTAXANTHIN ACCUMULATION IN *HAEMATOCOCCUS PLUVIALIS* *

LIU Jian-guo (刘建国), YIN Ming-yan (殷明炎), ZHANG Jing-pu (张京浦)
LIU Wei(刘伟), MENG Zhao-cai (孟昭才)
(*R&D Center of Marine Biotechnology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao* 266071, *China*)

Received July 26, 2000; revision accepted Feb. 24, 2001

Abstract This study on dynamic changes of culture color, astaxanthin and chlorophylls, inorganic N including $N\text{-}NO_3^-$, $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ in batch culture of *Haematococcus pluvialis* exposed to different additive nitrate concentration showed (1) ast/chl ratio was over 0.8 for brown and red algae, but was usually less than 0.5 for green and yellow algae; (2) $N\text{-}NO_3^-$, in general, was unstable and decreased, except for a small unexpected increase in nitrate enriched treatment groups; (3) measurable amounts of $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ were observed respectively with three change modes although no external nitrite and ammonia were added into the culture; (4) a non-linear correlation between ast/chl ratio (or color) changes and the levels of $N\text{-}NO_3^-$, $N\text{-}NO_2^-$, $N\text{-}NH_4^+$ in *H. pluvialis* culture; (5) up and down variation of the ast/chl ratio occurred simultaneously with a perceptible color change from yellow to brown (or red) when $N\text{-}NO_3^-$, $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ fluctuated around 30, 5, 5 μmol/L respectively; (6) existence of three dynamic modes of $N\text{-}NO_3^-$, $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ changes, obviously associated with initial external nitrate; (7) the key level of total inorganic N concentration regulating the above physiological changes during indoor cultivation was about 50 μmol/L; and (8) 0.5－10 mmol/L of nitrate was theoretically conducive to cell growth in batch culture.

Key words: *Haematococcus pluvialis*, nitrate, nitrite, ammonia, astaxanthin

INTRODUCTION

Over the past decade, *Haematococcus pluvialis* has been a subject of numerous investigations because its conspicuous red color points to its possibility as a hopeful regenerative bioresource of astaxanthin. Extensive study of culture factors (such as temperature, illumination, nutrient elements, and so on) determining astaxanthin accumulation, cell growth, cell forms and other related aspects yielded some complex results (Droop, 1954; Pringsheim, 1966; Donkin, 1976; Borowitzka et al., 1991; Bossiba and Vonshak, 1991; Bubrick, 1991; Yong et al., 1991; Grung et al., 1992; Kabayashi et al., 1993; Zlotnik et al., 1993; Hagen et al., 1994; Lee and Ding, 1994; Chaumont and Thepenier, 1995; Harker et al., 1995; Lu et al., 1995; Tan et al., 1995; Liu et al., 2000a, 2000b) showing inorganic nitrogen is probably crucial for this green alga. Previous studies on the effect of inorganic nitrogen on *Haematococcus* sp. mainly focused on the suitable N source, the favorable initial additive concentration and the preferential uptake. Results also showed a complex relationship between growth, cell yield, cell types and astaxanthin formation (Proctor, 1957; Stross, 1963; Borowitzka et al., 1991; Bossiba and Vonhsak, 1991; Jin et al., 1996). As there are few studies on the dynamic influence of the variable inorganic nitrogen during the culture process, our aim in this study is to gain better understanding of the influences of inorganic nitrogen on the culture results.

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* Contribution No. 4139 from Institute of Oceanology, Chinese Academy of Sciences.
Projects 39500114, 39970575, A/2786-1 and 9565 supported by NSFC, IFS (International Foundation for Sciences) and Sci-Tech Commission of Shandong Province respectively.

MATERIALS AND METHODS

Algae strain

Haematococcus pluvialis 712 was obtained from the Algal Collection Lab of the Institute of Hydrobiology, Chinese Academy of Sciences.

Culture conditions

Pre-N-starved *Haematococcus* were centrifuged and inoculated into fresh culture medium containing the following elements (mg/L): KH_2PO_4, 20; $MgSO_4 \cdot 7H_2O$, 100; $CaCl_2 \cdot 6H_2O$, 80; Na_2EDTA, 0.0198; $FeCl_3 \cdot 6H_2O$, 0.0244; $ZnCl_2$, 0.0041; H_3BO_3, 0.061; $CoCl_3 \cdot 6H_2O$, 0.0051; $CuSO_4 \cdot 5H_2O$, 0.006; $MnCl_2 \cdot 4H_2O$, 0.0041; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.038. KNO_3 ranging from 0.8 mg/L to 1.6 g/L (10 – 20000 μmol/L) was added to the medium with pH adjusted to 7.0. Cultures were placed in 1000 ml Erlenmeyer flasks with 600 ml medium at 24 – 30℃. The flasks were stoppered with cotton plugs and manually agitated 5 – 6 times every 24 hours. 12L/12D illumination of approximately 200μE/($m^2 \cdot s$) was provided from two sides of the flasks by 4 parallel fluorescent lamps mounted at the level of the cultures.

Samples collection

The samples for astaxanthin determination, chlorophyll examination, and inorganic N analysis were collected at intervals during the experiment, with syringes at about 11 A.M. when the cell division just ceased every day.

Astaxanthin and chlorophyll determination

Algae pelleted were frozen at −20℃ overnight and grounded in a tube-type glass mortar. Pigments containing astaxanthin and chlorophylls were extracted by acetone until colorless. Then, the extraction was centrifuged until it was transparent. Extinction of the supernatants was determined by spectrophotometer at 480, 645 and 663 nm.

The content of chlorophyll was given by the following formula (Arnon, 1949): chlorophyll (mg/L) = (20.2 $O.D_{645nm}$ + 8.02$O.D_{663nm}$) × the dilution ratio. Contribution of carotenoids (astaxanthin mainly) to the extinction at 480 nm was determined by the equation: $O.D_{480nm}^{ast} = O.D_{480nm} + 0.1140.D_{663nm} - 0.6380.D_{645nm}$. Therefore, astaxanthin content would be approximately estimated by the following equation (Davies, 1976; Sommer et al, 1991): astaxanthin (mg/L) = 4.6$O.D._{480nm}^{ast}$ × the dilution ratio.

$N\text{-}NO_2^-$, $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$ analysis

The culture samples collected at intervals were first centrifuged at 4000 r/min for 10 minutes. The residue was discarded and the supernatant was used to determine $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$. Two replicates were set up for each profile analysis in the experiment.

Distilled de-ionized water (DDW) was used for all the inorganic analyses and standard solutions. Ammonia-free H_2O was made by passing H_2O through a strong acid column of cation exchange resin (Dowex 50w X $4H^+$).

$N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ concentration in the culture were measured by using Shi's method (Shi et al., 1980), while $N\text{-}NH_4^+$ were measured by the method of Gao et al. (1980). The total inorganic

N in *Haematococcus* culture exposed to different nitrate treatment was expressed by the sum of NO_2^-, NO_3^- and NH_4^+.

RESULTS AND DISCUSSION

The color and astaxanthin/chlorophylls ratio

The varied colors of the culture were classified as red, brown, yellow and green. "+" and "−" showed the color level in each grade. "+" stands for more and "−" mean less. The color changes (Table 1) in each treatment group showed that low external nitrate reddened the algal culture, and high nitrate level led to yellow or green color. During the ongoing culture process, the brown low nitrate groups turned reddish, while the green high nitrate groups turned yellowish.

The colors of the cultures were depended on the content of two main pigments astaxanthin and chlorophylls, as well as their relative proportions. The green culture meant the reddish astaxantin was masked by the color of chlorophylls, and the red culture indicated more astaxanthin and less chlorophylls existed in *Haematococcus*. The ast/chl ratios of the culture under different treatment are listed in Table 2. Results showed that the lower the initial external nitrate the algae were exposed to, the more astaxanthin and less chlorophylls accumulated in the cells, so the ast/chl ratio was higher. On the other hand, high external nitrate led to less astaxanthin and much more chlorophylls, therefore to a low ast/chl ratio in the alga.

Table 1 The color changes of cultures under different nitrate treatment

Days	Treatment (the additive nitrate concentration, μmol/L)							
	10	50	100	500	1000	5000	10000	20000
1	*brown*	*brown*	brown$^-$	brown$^-$	brown$^-$	brown$^-$	brown$^-$	brown$^-$
3	*brown*	*brown*	*brown*	yellow	green	green	green	yellow
8	*brown*	*brown*	*brown*	*brown*	yellow	yellow$^-$	yellow$^-$	yellow$^-$
14	*red*	*red*	*red*	*brown*	yellow$^+$	yellow$^-$	yellow$^-$	yellow$^-$
20	*red*	*red*	*red*	*brown*$^+$	*brown*	yellow$^-$	yellow	yellow
27	*red*	*red*	*red*	*red*	*brown*	yellow	yellow$^+$	yellow$^+$
34	*red*	*red*	*red*	*red*	*red*$^-$	yellow	yellow$^+$	yellow$^+$
47	*red*	*red*	*red*	*red*	*red*	yellow$^+$	yellow$^+$	yellow$^+$

Table 2 The changes of astaxanthin/chlorophyll ratio with different nitrate treatment

Days	Treatment (the additive nitrate concentration, μmol/L)							
	10	50	100	500	1000	5000	10000	20000
5	*0.93*	*0.90*	*0.80*	0.54	0.48	0.48	0.47	0.67
8	*1.17*	*1.08*	*1.02*	0.67	0.44	0.33	0.39	0.54
15	*1.53*	*1.55*	*1.46*	*0.86*	0.62	0.31	0.42	0.60
27	*1.90*	*1.95*	*1.88*	*1.247*	*0.94*	0.36	0.34	0.37
48	*4.84*	*4.07*	*4.09*	*3.00*	*2.06*	0.55	0.55	0.52

Comparison of Table 1 and 2 indicated that the ast/chl ratio was usually higher than 0.8 for brown and red algae (data marked in italics), but was usually less than 0.5 for the green and yellow algae.

NO_3^- concentration

Although external NO_3^- added was confirmed, the NO_3^- concentration was unstable in the ongoing culture and always decreased (Table 3A) as a result of being absorbed by algae. Measurable decrease of NO_3^-

is shown in Table 3A (shaded data). An unexpected increase (data in white, Table 3A) was monitored in the high nitrate (>5000 μmol/L) treatment groups. If the initial additive nitrate was less than 1000 μmol/L, NO_3^- would decrease quickly to a limited level (ca. 2 – 3 μmol/L) during the culture period.

Table 3 The dynamic changes of NO_3^-, NO_2^-, NH_4^+ and total inorganic N (μmol/L) in *H. pluvialis* culture exposed to different nitrate treatment

	Time	Treatment (the additive nitrate concentration, μmol/L)							
	(d)	10	50	100	500	1000	5000	10000	20000
A	0.5	*4.7*	*24.5*	*55.2*	/	848	4633	9014	19806
	1	*2.2*	*4.4*	*3.0*	268	714	4457	8998	19376
NO_3^-	5	*1.3*	*2.2*	*1.7*	*15.1*	147	2734	7846	16498
(μmol/L)	8	*1.7*	*2.0*	*2.2*	*10.4*	57.2	3028	6694	16096
	15	*2.4*	*2.0*	*1.3*	*4.2*	*30.3*	3107	8241	18802
	27	*2.2*	*1.4*	*2.1*	*2.2*	*10.1*	2313	7224	17760
	48	*3.0*	*2.2*	*2.1*	*2.0*	*2.2*	929	7081	19156
B	0.5	*0.664*	*1.348*	*1.908*	1.45	1.535	1.452	1.286	1.742
	1	*0.353*	*0.373*	*1.016*	12.20	16.82	22.61	19.50	18.79
NO_2^-	5	*0.498*	*0.830*	*0.705*	9.418	29.85	29.13	23.77	22.57
(μmol/L)	8	*0.664*	*1.141*	*1.265*	8.795	28.80	32.43	24.95	24.44
	15	*0.581*	*0.726*	*0.830*	*3.630*	16.84	59.40	65.30	53.60
	27	*0.850*	*0.622*	*0.830*	*0.954*	*3.091*	346.4	309.1	179.0
	48	*0.208*	*0.145*	*0.203*	*0.519*	*0.416*	404.4	457.2	312.0
C	0.5	*2.600*	*2.238*	*3.324*	5.358	1.651	1.809	1.944	2.487
	1	*1.153*	*1.266*	*1.108*	8.841	7.665	18.36	13.95	12.41
NH_4^+	5	*2.577*	*1.560*	*1.515*	6.376	24.71	22.47	20.73	21.59
(μmol/L)	8	*1.017*	*1.108*	*1.131*	5.222	21.25	25.77	20.57	21.71
	15	*2.509*	*1.356*	*1.108*	*2.600*	8.612	61.47	61.24	47.61
	27	*1.850*	*0.880*	*1.467*	*1.218*	*3.244*	273.0	281.8	156.8
	48	*0.857*	*0.857*	*0.880*	*0.745*	*0.812*	273.0	303.0	170.1
D	0.5	*8*	*28*	60	/	851	4636	9017	19812
	1	*4*	*3*	*5*	289	739	4498	9031	19407
Total	5	*4*	*5*	*4*	*31*	202	2789	7889	16522
inorganic	8	*3*	*4*	*5*	*24*	107	3086	6740	16142
N	15	*6*	*4*	*3*	*10*	66	3228	8368	18903
(μmol/L)	27	*5*	*3*	*4*	*4*	*17*	2932	7795	18100
	48	*4*	*3*	*3*	*3*	*3*	1606	7841	19638

Comparison of Tables 1, 2 and 3A showed that the astaxanthin accumulation was more dependent on the actual NO_3^- level in the culture than the initial additive nitrate. The up and down variations of ast/chl ratio was accompanied by NO_3^- fluctuation around 30 μmol/L, and a perceptible color change from yellow to brown or red (see data in italics in Tables 1, 2 and 3A).

NO_2^- and NH_4^+ concentration

Although no external nitrite and ammonia were added into the culture in this experiment, certain amount of NO_2^- and NH_4^+ was also measured with three change modes, respectively (Table 3 B and C). Firstly, both NO_2^- and NH_4^+ content were limited to minimum level (ca. 0 – 2 μmol/L) but still decreased in nitrate deficient treatment groups (10 – 100 μmol/L). Secondly, both NO_2^- and NH_4^+ content increased linearly from nearly 0 to ca 460 and 300 μmol/L, respectively, in ni-

trate enriched treatment groups (5000 – 20000 μmol/L). In the third mode, both NO_2^- and NH_4^+ first increased, then decreased to minimum content (shaded data in Table 3B and C) in medium level nitrate (500 – 1000 μmol/L) treatment groups.

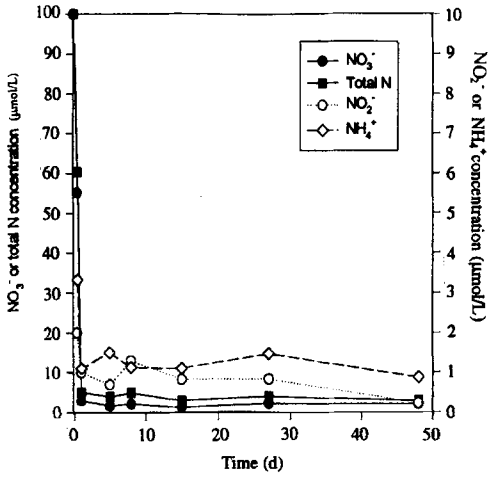

Fig.1 The dynamic changes of inorganic N in *H. pluvialis* culture exposed to low (0.1 mmol/L) nitrate treatment

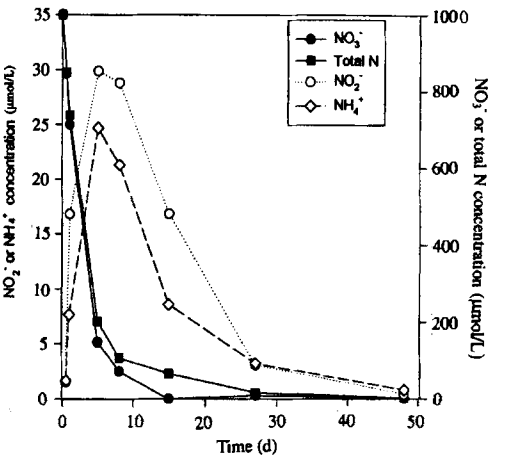

Fig.2 The dynamic changes of inorganic N in *H. pluvialis* culture exposed to medium (1 mmol/L) nitrate treatment

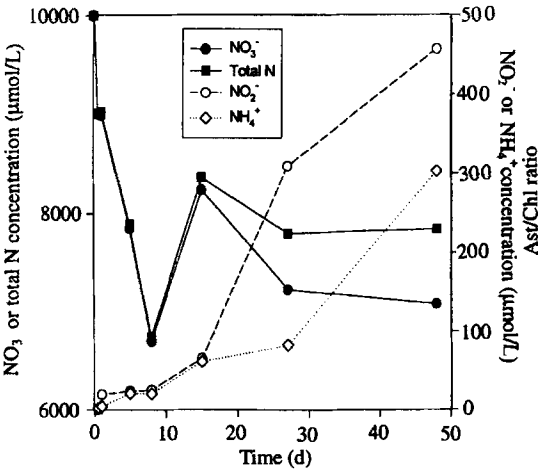

Fig.3 The dynamic changes of inorganic N in *H. pluvialis* culture exposed to high (10 mmol/L) nitrate treatment

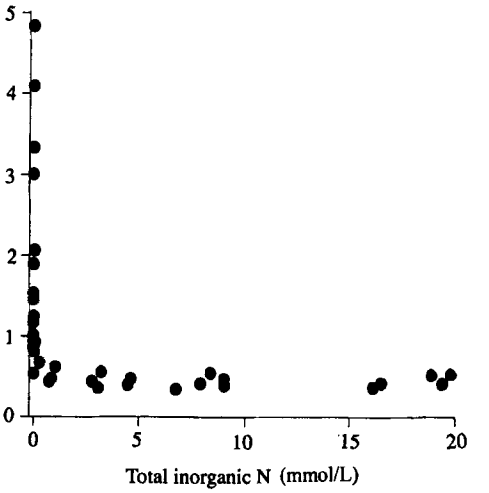

Fig.4 The relationship between total inorganic N and the ast/chl ratio

Comparison of Tables 1, 2, 3B and C, showed that a non-linear correlation between the ast/chl ratio (or color) changes and the NO_2^- (or NH_4^+) level existed in the *Haematococcus* culture. The brown and red culture (or ast/chl ratio > 0.8) had small content (< 2 – 3 μmol/L) of NO_2^- (or NH_4^+) (data in italics in Tables 1, 2, 3 B and C), while the yellow and green culture (or cul-

ture with ast/chl ratio < 0.5) had high content (>5 μmol/L) of NO_2^- (or NH_4^+).

Three dynamic modes of inorganic nitrogen changes

Three modes of N-NO_3^-, N-NO_2^- and N-NH_4^+ changes obviously associated with initial external nitrate, were observed. When the additive external nitrate was low (< 100 μmol/L), NO_3^- would be effectively consumed to its minimum level within one day due to the algal's strong absorptive ability, and not much N-NO_2^- and N-NH_4^+ were produced (Fig. 1). N-NO_3^- was partly consumed by the algae in nitrate enriched culture (>5000 μmol/L), at the same time that certain amount of N-NO_2^- and N-NH_4^+ was produced (Fig. 3). If the additive external nitrate ranged from 500 to 1000 μmol/L, N-NO_3^- was preferentially consumed, and the N-NO_2^- and N-NH_4^+ produced at the initial stages were then consumed in the ongoing culture (Fig. 2).

Algae, such as *Chlorella*, absorb ammonium in the presence of nitrate and exhaust the supply of ammonium before using nitrate (Cramer and Myers, 1948; Pratt and Fong, 1940). Our results showed that *H. pluvialis*, like certain related genera in high plants, can absorb nitrate preferentially when presented ammonium and nitrite, which support some former results of study on this alga (Proctor, 1957; Stross, 1963).

The relationship between total inorganic N in the culture and the ast/chl ratio

The total inorganic N (NO_3^- plus NO_2^- and NH_4^+) in the *H. pluvialis* cultures exposed to different nitrate treatment groups are shown in Table 3D. The large net and relative consumption of total inorganic N by the algae were monitored in medium nitrate treatment groups (500 – 1000 μmol/L), which theoretically showed that certain inorganic N concentration (within 0.5 – 10 mmol/L) were conducive to cell growth in batch culture.

Although more net nitrate was consumed when the alga was cultured at 5000 – 10000 μmol/L, which is similar to the obtained favorable nitrate concentration for cell growth (Borowitzka et al., 1991), the relative consumption is still low in high nitrate treatment. Therefore, low N concentration of 500 – 1000 μmol/L is suggested for commercial culture. The reasons are high efficiency of nitrate uptake, less resulting nitrate enriched sewage water, fast cell growth, rapid use up of nitrate, easy transformation from motile cells to non-motile cells, enhancement of astaxanthin accumulation, facilitation of algal harvesting, more economic culture, and reduced formation of ammonia nitrogen. Ammonia is regarded as the least suitable N source for *Haematococcus* growth as its use results in reduced maximum cell number (Borowitzka et al., 1991).

Based on the above results of our study on ast/chl ratio, the culture color, the measured total inorganic N in the culture and the external N added, we can summarize that the ast/chl ratio (or culture color) was largely dependent on the total inorganic N (NO_3^- plus NO_2^- plus NH_4^+) concentration in the culture, but not on the initial N added. This relationship can be further shown in Fig. 4 where the inflection of the ast/chl ratio vs total inorganic N curve is at ast/chl ratio of 0.5 and total inorganic N of 50 μmol/L.

In brief, 50 μmol/L of total inorganic N, commonly considered as N deficient condition, is suggested as a key N concentration in theoretical study and commercial culture for astaxanthin, because it regulated the cell growth, cell transformation and astaxanthin accumulation in *H. pluvialis*.

ACKNOWLEDGEMENTS

We wish to express our thanks to the National Natural Science Foundation of China (NSFC), Ministry of Education of China and International Foundation for Sciences (IFS) for their financial support.

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