

## Some growth metabolites of two cyanobacteria species as affected by mineral nutrition

\*<sup>1</sup>El-Sayed, A.B; <sup>1</sup>El Fouly, M.M; <sup>2</sup>Ghazy, S.M; <sup>2</sup>El Semary, N.A and <sup>1</sup>Hassoub, M.A

<sup>1</sup> Fertilization Technology Department, National Research Centre, Cairo-Dokki, Egypt

<sup>2</sup>Botany and Microbiology Department, Faculty of Science, Helwan University, Egypt  
[bokhair@msn.com](mailto:bokhair@msn.com)

**Abstract:** Two blue green algae species belonging to Cyanophyta (*Anabaena flos-aquae* and *Calothrix* sp.) were exposed to different nutrient concentrations of nitrogen, phosphorous and iron to claim the biological effect of such nutrients on pigmentation, peptide nitrogen and carbohydrates. Growth media of BG-II was used for vegetative control growth, while experimental growth was performed under different concentrations of nitrogen, phosphorous and iron. The applied concentrations were 0.25, 0.5, 1.0 and 2.0 fold of the original BG-II growth medium. Growth parameters were peptide nitrogen, total sugars and pigments fraction including chlorophyll-a, total carotenes and phycobiliproteins. Nutritional regime affected the evaluated parameters and seems to be algal dependent, Peptide nitrogen was linearly stimulated due to the elevated nutrient, however the increment were found towards Fe, P, N. Total sugars were more affected, however both algae mostly represented different manner especially with nitrogen and iron treatment. On the other hand, phosphorous cultures represented the same for both algae. Chlorophyll-a and phycobiliproteins were found in relative to the rise of all nutrient used, although carotenes exhibited a variable response under different nutrient used or their concentration. [Nature and Science 2010;8(12):20-28] (ISSN: 1545-0740).

**Key words:** *Anabaena*, *Calothrix*, Mineral Nutrition, Pigments, Carbohydrates, Protein

### 1. Introduction

Microalgae produce of wide variety of biomaterials, including chlorophyll. Most algae cultured under optimum condition contained about 4% of total chlorophyll on dry weight basis. Chlorophyll provides a chelating agent activity which can be used in ointment, treatment for pharmaceutical benefits especially liver recovery and ulcer treatment. Besides that, it repairs cells, increases hemoglobin in blood and faster the cell growth (Puotinen, 2009).

*Spirulina* a well-known blue green alga is still used in food supplements due to its excellent nutrient compounds and digestibility (Kumar *et al.*, 2005). Besides higher content of protein (60–70 %), *Spirulina* also contains a rich source of vitamins, especially vitamin B12 and pro-vitamin A ( - carotene) and minerals (Thajuddin and Subramanian, 2005).

It has been conclusively shown that iron chelating properties is widespread among freshwater and marine cyanobacteria and that some of these iron chelators function as siderophores (Wilhelm and Trick, 1994 & Kerry *et al.*, 2008). Siderophores are low-molecular weight, high-affinity Fe<sup>+3</sup> binding ligands secreted by bacteria under conditions of iron stress to scavenge and transport iron. Cyanobacteria have also been shown to produce low-molecular weight (400 to 1200 Da) ferric-specific chelators (siderophores) under iron-limiting culture conditions (Trick and Kerry, 1992 & Wilhelm and Trick, 1994).

Siderophores bind to Fe<sup>+3</sup> to form a ferri-siderophore complex which facilitates the transport of ferric ions into cells during periods of iron deficiency (Matzanke, 1991).

Different small and simple organic molecules such as hydroxamic acids or citrate for iron chelating. Schizokinen, a citrate derivative siderophore from the freshwater cyanobacterium *Anabaena* PCC 7120 (Goldman *et al.*, 1983); from the marine cyanobacterium, *Synechococcus* PCC 7002 (Ito and Butler, 2005) have been detected. It has been suggested that due to amphiphilic structure of these siderophores they can fix into the membrane of cyanobacterium and thus prevents its loss by dilution in marine environments (Martinez *et al.*, 2006). Another two complex siderophores, anachelin H and anachelin 1, were isolated from biofilm-forming cyanobacterium, *A. cylindrica* (Beiderbeck *et al.*, 2000). It confines iron from solid minerals of marine as well as freshwater environments (*e.g.*, iron oxide hydrates), stones and rocks. Siderophores must recognize, bind and sequester iron from solid minerals. It has been shown that anachelin chromophore recognizes metal oxide surfaces as found in these minerals (Gademann, 2006; and Zürcher *et al.*, 2006).

The photochemistry of siderophores produced by marine bacteria, as well as several isolated from cultures of the marine cyanobacterium

*Synechococcus* was studied on the basis of their characteristic  $Fe^{+3}$  binding functionalities: hydroxamate, catecholate, and  $\alpha$ -hydroxy carboxylate groups and it was pointed out that siderophore photoreactivity is largely determined by the nature of the  $Fe^{+3}$  binding groups (Barbeau *et al.*, 2003). The thermodynamic stability constants for the complexation of iron<sup>+3</sup> with catecholate-type siderophores isolated from the marine bacterium, *Alteromonas luteo-olivacea* and cyanobacterium, *Synechococcus* sp. PCC 7002 was estimated and it was suggested that catecholate siderophores may play a role in the solubilization and biological uptake of iron in the marine environment (Lewis *et al.*, 1995). However, the molecular mechanism of Fe acquisition employed by open ocean cyanobacteria has yet to be established. It has been reported that both inorganic  $Fe^{+3}$  in the presence of oxygen, and humic  $Fe^{+3}$  stimulates lipid peroxidation, trigger or stimulate the release of chelators from the green algae *Scenedesmus* & *Chlorella*; red alga *Porphyridium* and cyanobacteria *Arthonema*, *Arthrospira* (Benderliev, 1999).

The iron acquisition by siderophores is supposed to be of great importance in molecular understanding of iron uptake by marine and freshwater organisms (Gademann and Portmann, 2008). Siderophores recognize and bind to iron oxide and promote bio-film formation by cell adhesion to metal oxide such as titanium oxide surfaces very efficiently and a novel biomimetic strategy based on the iron chelator anachelin have recently been developed for surface modification that exploits the evolutionary optimized strong binding affinities of iron chelators (Zürcher *et al.*, 2006 and Gademann, 2007).

Blue-green algae (also called cyanobacteria) have an impressive ability to colonize infertile substrates such as volcanic ash (Brock, 1973); and desert sand and rocks (Friedmann & Galun, 1974; Metting, 1991 and Painter, 1993). Growth without exogenous combined nitrogen is possible because heterocystous, filamentous genera are autonomous (non-symbiotic) nitrogen-fixers (Bothe, 1982). The ability to derive phosphate and micro-nutrients from insoluble minerals is, however, understood only in outline. In fertile soils, mineral diagnosis, with release of plant nutrients in soluble form, is brought about by humic acids, produced by the decay of higher plant material (Kononova, 1961). The mechanism entails the sequestration of multivalent metal cations such as Ca, Al and Fe (Schnitzer, 1978). Since the algae will grow on volcanic ash, which contains no organic matter, it must be presumed either that the living cells synthesis a substance that functions in the same way, or that it is formed upon the death and decay of the

algal trichomes (Verma and Martin, 1976 & Zunino and Martin, 1977).

Nutrient manipulation, particularly reduction of nutrient inputs, should be viewed as the best approach for turn over growth of algae as well as the massive accumulation of certain metabolites if needed by such approach. (Lembi, 2000). Many studies early showed the strong correlation between total phosphorus (TP) and planktonic algal biomass (Dillon & Rigler, 1974; Jones & Bachmann, 1976; Schindler, 1978 and Prepas & Trew, 1983). The positive relationship between chlorophyll-a concentrations and TP is a commonly used tool to predict water quality and trophic status (Vollenweider, 1969; Dillon & Rigler, 1974 and Dillon *et al.*, 1988).

N limitation, as well as P limitation, has been implicated in the regulation of filamentous algal growth (Spencer and Lembi, 1981; O'Neal *et al.*, 1985 and Dodds & Gudder, 1992). In addition to the amount of phytoplankton biomass that is produced with P or N additions, another consideration is species composition, particularly in relation to nutrient ratios. (Tilman and Kiesling, 1984). N-fixing cyanobacteria (*Anabaena*, *Aphanizomenon*, and others), which fix atmospheric  $N_2$  when the water becomes N-depleted, become dominant; particularly during the summer months. (Schindler, 1977; Smith, 1983; Schelske and Stoermer, 1971; Schelske, 1975). For this reason, the emphasis on nutrient removal for generally improving water quality has been placed on P rather than on N. In fact, increasing the N:P ratio stimulated the growth of non-nitrogen fixing cyanobacteria such as *Lyngbya*, *Oscillatoria*, and *Chroococcus* (Havens and East, 1997). Since uptake is the main mechanism of nutrient removal by microalgae, the microalgal population growth rate directly affects the nutrient removal rate. Meanwhile, nitrogen and phosphorus could be simultaneously utilized and removed efficiently only if the N/P ratio in wastewater is in a proper range (Xin *et al.*, 2010). Thus, the current work was carried out to determine the growth behaviour (as some growth metabolites) of two blue green algae species grown under different concentrations of nitrogen, phosphorous and iron.

## 2. Materials and Methods

### 2.1. Algae and growth conditions

Two blue green algae species were used to fulfill the current study namely *Anabaena flos aquae*, and *Calothrix* sp. PCC7601. BG-II growth medium (Stainer *et al.*, 1971) was used in original form. *Anabaena flos-aquae* was grown at 20  $\mu$ e of light intensity, while *Calothrix* sp. was grown at 15  $\mu$ e. White color was provided from fluorescent lamps.

Growth and treatments were employed with three replicates of each treatment. Concerning nutritional treatments, 0.25, 0.50 and 2.0 folds of nitrogen, phosphorous and iron concentrations were separately tested against control concentration of BG-II (expressed as 1.0).

## 2.2. Measurements and analysis

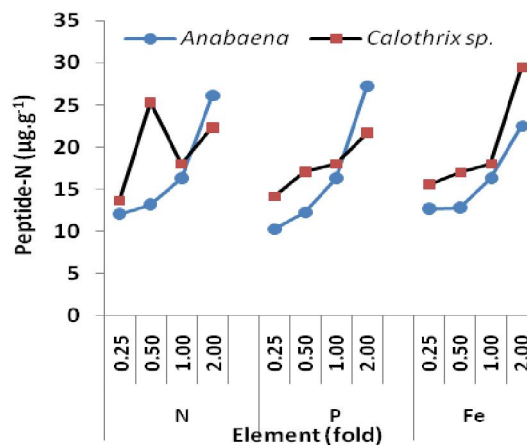
Protein was determined on ethanol extract according to Lowry *et al.* (1951). Total sugars were determined by anthrone technique as described by Umbriet *et al.* (1959). Chlorophyll-a and total carotene contents was determined in 80% (v/v) acetone extract. Chlorophyll-a and total carotene concentrations were calculated according to McKinney (1941) Pheycobiliproteins were measured according to Bennet and Bogard (1973).

## 3. Results and discussion

### 3.1. Effect of nutrients on peptide nitrogen

Three lines of comparison should be put in consideration. The first is the different effect of three examined nutrients (N,P and Fe). The second is the concentrations applied of such nutrients. Third and final is the alga under investigation, in spite of the measured parameters (Protein, carbohydrates and pigments). Concerning the nutrient used, nitrogen was early considered as the main effective nutrient on protein formation and surpasses any other nutrient in this action. Both algae represented the same response pattern against the protein accumulation under different nutrient concentrations. As for nitrogen concentrations, the difference was observed between both algae on the accumulated protein content only at minor nitrogen concentration (0.5N). Maximum protein yield was observed with super-optimal concentrations of nitrogen (2.0N) in *Anabaena*, while protein content was found in association with the rise of nitrogen concentrations. Such maximum was observed under sub-optimal nitrogen concentration (0.5N) with *Calothrix* sp, however super-optimal nitrogen concentration also surpasses the control content. The obtained data was found in the harmony of the base of N:P ratio, where changing of nitrogen concentration led to the change of this ratio. Under sever nitrogen depletion, phosphorous became the stressful nutrient and this might be led to the decreases on protein accumulation. This could be supported by the next data obtained from the moderate depleted nitrogen concentration (0.5N) with *Calothrix* sp. Here, the stress effect of phosphorous might be downed. Increasing of nitrogen concentration up to the control content (2.0N) revealed the deficient of initial nitrogen content from BG-II to both the examined algae. In general,

*Anabaena* seems to be more responses to accumulate protein rather than *Calothrix* sp under depleted and higher nitrogen concentrations.



**Fig. 1.** Peptide nitrogen content ( $\mu\text{g}\cdot\text{g}^{-1}$ ) of *Anabaena fols-aquae* and *Calothrix* sp. under different concentrations of nitrogen, phosphorous and iron concentrations (0.25, 0.5, 1.0 and 2.0 fold of the original BG-II growth media).

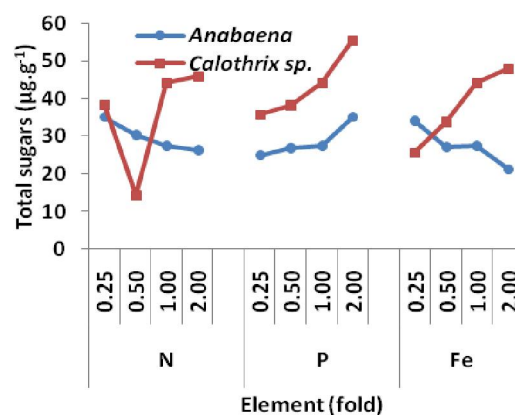
On the contrast with nitrogen treatment results, phosphorous more affected protein content in both algae as compared with nitrogen. A proportional increases were observed due to the increasing the phosphorous concentrations in growth media of both algae. Under low phosphorous concentrations, *Calothrix* seems to be more affected than *Anabaena* that more stimulated under super-optimal concentrations. This might be attributed to the genomic base of *Anabaena* to fix the atmospheric nitrogen under condition of nitrogen limitation and utilize it to complete their life cycle. Under such conditions, *Calothrix* sp. surpasses *Anabaena*, where peptide nitrogen was increased under moderate, normal and super optimal concentration of nitrogen (*i.e.*, 0.5, 1.0 and 2.0N). This might be goes back to the original biochemical composition of both algal species. The effect of super-optimal nitrogen concentration could be ascribed to the accompanied potassium ions liberated from nitrate consumption. However the initial amount on protein content was approximately the same, incubation under different nitrogen concentrations increased the initial content at the end of incubation.

The effect of phosphorous on growth and other behaviour of blue green algae; in many ways; is nitrogen dependent. Many studies claimed that nitrogen phosphorous ratio is more important in algal

growth rather than element concentration. In addition to the amount of phytoplankton biomass that is produced with P or N additions, another consideration is species composition, particularly in relation to nutrient ratios. When sufficient silicon (Si) and N are available in relation to P (high Si:P and N:P ratios), diatom growth appears to be favored (Tilman and Kiesling, 1984). These conditions are typical of spring periods in temperate lakes following turnover or when sediment deposition occurs with spring rains. In late spring or early summer, green algae may dominate over diatoms as Si concentrations decrease lower Si:P ratios (Sommer, 1983).

From a management standpoint, the most critical nutrient ratios are low N:P or Si:P. These generally occur under conditions of excessive P loading, and it is under these circumstances that N-fixing Cyanobacteria (*Anabaena*, *Aphanizomenon*, and others), which fix atmospheric  $N_2$  when the water becomes N-depleted, become dominant (Schindler, 1977 and Smith, 1983), particularly during the summer months. For example, the shift from high Si:P and N:P ratios to low ratios was concomitant with the shift in dominance from diatoms to cyanobacteria (Schelske and Stoermer, 1971 & Schelske, 1975). For this reason the emphasis on nutrient removal for generally improving water quality has been placed on P rather than on N. Another approach for maintaining a high N:P ratio is to increase N rather than to decrease P. In fact, several researchers (Leonardson and Ripl, 1980 & Smith, 1983) suggested that N removal could be counterproductive and that N addition might actually be helpful in increasing populations of green algae or diatoms in relation to cyanobacteria. Barica *et al.* (1980) added N to ponds with low N:P ratios to see if it could reduce the incidence of cyanobacterial blooms, particularly blooms of *Aphanizomenon* that were causing fish kills when they crashed. The initial N:P ratios in these ponds were around 4 to 5. Schindler (1977) found that cyanobacteria dominate when N:P ratios dropped from 15 to 5. When low amounts of N were added ( $0.1 \text{ g Nm}^{-3} \cdot \text{d}^{-1}$ ) prior to bloom formation (but not during the bloom) or when high amounts ( $1.0 \text{ g Nm}^{-3} \cdot \text{d}^{-1}$ ) were added during the bloom, a shift from *Aphanizomenon* to green algae and *cryptomonads* was occurred. Although similar results were reported by Stockner and Shortreed (1988), the general consensus is that, when possible, it is much better to reduce P concentrations than to elevate N concentrations. In fact, increasing the N:P ratio stimulated the growth of non-nitrogen fixing cyanobacteria such as *Lyngbya*, *Oscillatoria*, and *Chroococcus* (Havens and East, 1997). Elser *et al.*

(1990) suggested that both P and N potentially limited algal growth. They found little support for P alone as a causative factor. However, they recommended that efforts should concentrate on P reduction because it is easier to achieve from a technical standpoint than N reduction. Clearly where N fixation by planktonic cyanobacteria is a response to N reduction, P should be the more reliable means to lower algal biomass. The same general approach is used for the control of filamentous algae and *Chara*, although the situation regarding specific nutrient ratios and the target amounts is far from clear. Furthermore, low N: P ratios enhanced growth and geosmin production, thus, treatment of aquatic environment with high N: P ratios would exclude this species. Phosphorus and nitrogen levels in aquatic habitats represent the main factors promoting the odor problem caused by this cyanobacterium. Loadings at N:P ratios between 10 and 25 increased algal biomass with a summer community dominated by  $N_2$ -fixing *Anabaena circinalis*; increasing the N:P ratio to 35 retained the higher biomass, but shifted the community dominance to small-celled *Synechococcus* spp. Eutrophic lakes with low N:P ratios favor blooms of  $N_2$ -fixing cyanobacteria; hence, nutrient cycling within these lakes is closely coupled these organisms (Schindler, 1977 and 1985).



**Fig. 2.** Total sugars content ( $\mu\text{g.g}^{-1}$ ) of *Anabaena fols-aequae* and *Calothrix* sp. under different concentrations of nitrogen, phosphorous and iron concentrations (0.25, 0.5, 1.0 and 2.0 fold of the original BG-II growth media).

Algal species have different micronutrient requirements, such as Si, Mg, Ca, Fe, Mo, and Se. Diatom dominance in phytoplankton assemblages is dependent on recycled Si following spring turnover;

lakes with lower Si levels may lack *aspringtime* diatom pulse altogether. The haptophyte *Chrysochromulina breviturrita* develops large populations in dilute lakes undergoing the early stages of acidification (Nicholls *et al.*, 1982). Its requirement for Se and  $\text{NH}_4$ , and its inability to use  $\text{NO}_3$  favor its success in oligotrophic lakes within the pH range 5.5–6.5 (Wehr and Brown, 1985 and Wehr *et al.*, 1987). Nutrient requirements may have important interactions, for example, Mo, which is an essential micronutrient for some cyanobacteria as a co-factor for  $\text{N}_2$  fixation; its assimilation may be inhibited by elevated  $\text{SO}_4$ , which is common in saline lakes (Howarth and Cole, 1985 and Marino *et al.*, 1990).

Since uptake is the main mechanism of nutrient removal by microalgae, the microalgal population growth rate directly affects the nutrient removal rate. Meanwhile, nitrogen and phosphorus could be simultaneously utilized and removed efficiently only if the N/P ratio in wastewater is in a proper range.

### 3.2. Effect of nutrients on total sugars

The other determined growth-related metabolite namely total sugars exhibited a different manner. Under different nitrogen concentrations (0.25-2.0N), *Calothrix* sp. surpasses *Anabaena* for total sugars accumulation. As for the different used concentrations, low nitrogen (0.25 and 0.50N) represented different increases in *Anabaena*, however extreme N depletion was resulted the maximum. Super-optimal nitrogen concentration also increased the inhibitory effect of sugars n was observed with *Calothrix* sp. that exhibited a proportional increases on sugar accumulation due to the degree of nitrogen increment dose, except the sub-optimal nitrogen concentration (0.5N) that exhibited a sharp decrease.

It may be concluded that increasing of nitrogen concentration, however increased total sugars of *Calothrix* sp. they decreased such content in *Anabaena*. On the other hand, phosphorous increased the accumulation of total sugars of both algae, where all concentrations (0.25 to 2.0P) resulting in a proportional increases. Increasing of phosphorous might be disturbed the N:P ratio on the expense of nitrogen. The low nitrogen content is expected to decrease protein accumulation, so accumulation of carbohydrate and lipids took place.

On the contrary, different iron concentrations resulted in two different patterns on sugar accumulation due to the examined algae. Sugar accumulation was drastically increased the sugars content of *Calothrix* sp., while they decreased such content of *Anabaena*. The effect of iron on growth media was earl described as they damaged algal cells

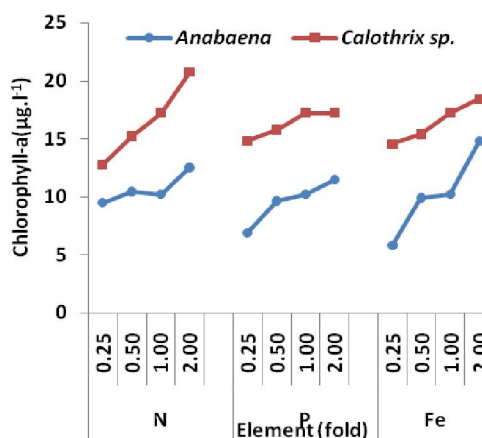
through the production of OH free radical (Fenton reaction). The net gain of this action is to destroy chlorophyll, protein and finally cells tended to accumulate large quantities of different metabolites than protein including lipids, carbohydrates and carotenoids pigments.

The obtained data obviously showed that the *Anabaena* surpasses *Calothrix* sp. in peptide nitrogen accumulation under nitrogen and phosphorous treatments. Otherwise, iron more stimulated by *Calothrix* sp. rather than *Anabaena*. As for total sugars, *Calothrix* resulted the opposite manner except under iron extra supplementation growth.

### 3.3. Effect of nutrients on pigmentation

#### 3.3.1. Chlorophyll-a

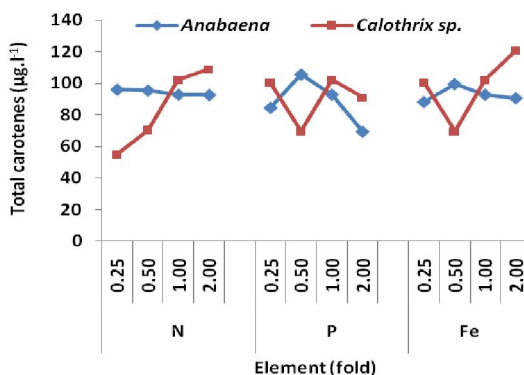
The initial content of *Calothrix* sp. seems to be higher than *Anabaena* and both of them the same response against on chlorophyll content nutrients supplementation. In all cases, chlorophyll content was increased toward the extra nutrient supplementation.



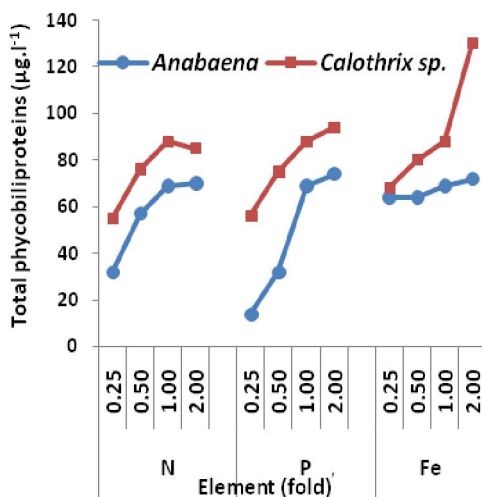
**Fig. 3.** Chlorophyll-a content ( $\mu\text{g.l}^{-1}$ ) of *Anabaena fols-aquae* and *Calothrix* sp. under different concentrations of nitrogen, phosphorous and iron concentrations (0.25, 0.5, 1.0 and 2.0 fold of the original BG-II growth media).

Among the different examined nutrients respected to the grown algae, nitrogen seems to be more effective in *Calothrix* sp. case. Phosphorous and iron resulted in slight and gradual increases with *Calothrix* sp. In addition, both nitrogen and phosphorous had the same enhancing effect on chlorophyll accumulation of *Anabaena*, however the lower concentration of nitrogen (0.5N) resulted in a slight increase as compared with control culture.





**Fig. 4.** Carotenenes content ( $\mu\text{g.l}^{-1}$ ) of *Anabaena fols-aquae* and *Calothrix* sp. under different concentrations of nitrogen, phosphorous and iron concentrations (0.25, 0.5, 1.0 and 2.0 fold of the original BG-II growth media).



**Fig. 5.** Total phycobiliproteins content ( $\mu\text{g.l}^{-1}$ ) of *Anabaena fols-aquae* and *Calothrix* sp. under different concentrations of nitrogen, phosphorous and iron concentrations (0.25, 0.5, 1.0 and 2.0 fold of the original BG-II growth media).

### 3.3.2. Carotenenes

Similar to chlorophyll, carotenenes content was differed due to the examined alga. Increasing of nitrogen concentration led to the increase of carotenenes content of *Calothrix* sp., however led to a slight decrease in *Anabaena*. The role of nitrogen in carotene accumulation is completely understood.

Decreasing of nitrogen supplementation should be led to carotenenes increasing.

The increasing of carotenenes under high nitrogen concentration might be attributed to the changing of N:P ratio. Consequently, carotenenes were decreased in *Anabaena* under lower and hyper-optimal concentrations, except those received the moderate concentration (0.5P); however, such concentration (0.5P) drastically inhibits the accumulation of carotenenes in *Calothrix* sp. The similar variable trend was also observed with both algae as they treated with different iron concentrations. Except control treatment (1.0Fe); all iron concentrations inhibit the accumulation of carotenenes in *Anabaena* with complete different pattern with *Calothrix* sp. Minor, control and higher concentrations of iron (0.25, 1.0 and 2.0Fe) enhanced the carotenenes content, while the lower concentration (0.5Fe) inhibited it.

### 3.3.3. Phycobiliproteins

A singlet response pattern of both isolates on phycobiliproteins as they incubated under the aforementioned nutritional regime. In general, the phycobiliprotein content was proportionally associated with nutrients reduction and enrichment. In both isolates, nitrogen decreasing as well as extra nitrogen concentrations (0.5 and 2.0 N) decreases the phycobiliprotein content. Otherwise, the moderate nitrogen increase (2.0N) resulted the same of control culture.

This finding was found in the harmony of the physiological function of such pigments, since phycobiliproteins; the accessory pigment; are required to achieve photosynthesis under stress conditions including starvation and over doses of nutrients. Thus, such pigments are expected to increase. In most blue green algae case, the ability of atmospheric nitrogen utilization might be prevent their dominance.

## 4. Conclusion

The effect of nitrogen, phosphorous and iron on some growth metabolites including peptide nitrogen, total sugars and pigments could be served as alga dependent rather than both nutrient kind and their concentration. However, both algae sometime exhibited the same growth pattern, they varied on their initial or/and the net experimental gain.

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