

## INFLUENCE OF HUMIC ACID ON PIGMENTS, METABOLITES AND THE ACTIVITIES OF ENZYMES OF NITROGEN METABOLISM

\*Hamed M El-Shora, \*Mohammed I Abdelhamid, \*\*Mohamed Z. Nassar, and \*Samir Abdel-Kader

\*Botany Department, Faculty of Science, Mansoura University, Egypt

\*\*National Institute of Oceanography and Fisheries

shoraem@yahoo.com

### ABSTRACT

*In this investigation *Chlorella homosphaera* and *Scenedesmus quadricauda* were treated with humic acid (30 mgL<sup>-1</sup> and 50 mgL<sup>-1</sup> for *S. quadricauda* and *C. homosphaera*, respectively) followed by determination of Chl a, Chl b, total Chl, total carotenoids and total pigments. Also the total free amino acid, total carbohydrate, total lipid, total phenol contents were determined. In addition, the activities of nitrate reductase, nitrite reductase, glutamine synthetase as well as urease were estimated. The obtained results reveal that treatment of both algae with humic acid resulted in the increase of total chlorophyll, total carotenoids and the total pigment content. In addition, the treatment of both algae with humic acid enhanced the activities of nitrate reductase (NR, EC: 1.7.1.1), nitrite reductase (NiR, EC: 1.7.2.1), glutamine synthetase (GS, EC: 6.3.1.2) and urease (EC: 3.5.1.5) with different rates. Thus, the present results suggest application of humic acid in fish farming to enrich the farming with algal species which are considered as feed for fish and it is economic application.*

**Keyword:** *Chlorella homosphaera, Scenedesmus quadricauda, Humic acid, Metabolites, Enzyme activity.*

### INTRODUCTION

Algae are the source for about half of the O<sub>2</sub> production to the atmosphere (Gibson *et al.*, 1990). Microalgae play a vital role in the rearing of aquatic animals like mollusks, shrimp, fish, and are of strategic interest for aquaculture (Benemann, 1992; Muller-Feuga, 2000). Phytoplankton represents the base of the entire aquatic food chain, supporting the production of 100 x 10<sup>6</sup> t of fish per year

(Longhurst *et al.*, 1995; Pauly and Christensen, 1995).

The main applications of microalgae for aquaculture are related to nutrition, being used fresh as a sole component or as a food additive to basic nutrients. There are a number of different applications in which microalgae are utilized in aquaculture, their main applications, however, being related to nutrition,

the basis of the energy flow through the aquatic grazing food chain (De Pauw and Per-Soone, 1988).

The nutritional value of microalgae depends mainly on their cellular structure and chemical composition, which are influenced by culture conditions (Brown *et al.*, 1997, Uriarte *et al.*, 2004). Microalgae should be nontoxic, possess the essential nutritive constituents, of proper size to be ingested and should have a digestible cell wall to make the nutrients available (Marshall *et al.*, 2010).

Humic acid is a commercial product contains many elements which improve the soil fertility, increase the availability of nutrient elements and consequently affect plant growth and yield. Humic acid particularly is used to remove or decrease the negative effects of chemical fertilizers and some chemicals from the soil. The major effect of humic acid on plant growth has long been reported (Pal and Sengupta, 1985; David *et al.*, 1994; Hartwigson and Evans, 2000).

The presence of HS has been shown to stimulate photosynthesis in the green alga, *Pseudokirchneriella subcapitata*, by reducing the toxicity of heavy metals (Koukal *et al.*, 2003).

Nitrate reductase is a regulatory enzyme restricting the rate of nitrate accumulation. NR plays a central role in primary metabolism and exhibits complex regulation mechanisms for its catalytic activity (Kaiser and Huber, 2001). Nitrite reductase catalyzes the assimilation of nitrite to ammonium. The enzyme is

located in the chloroplast of green algae (Lopez-Ruiz *et al.*, 1991). GS is an enzyme that plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine (El-Shora and Ali, 2011). Urease exercise a single catalytic function, that is the hydrolysis of urea ( $H_2N-CO-NH_2$ ), its final products being ammonia and carbonic acid (El-Shora, 2001).

The aim of the present work was to investigate the effect of humic acid on pigments, metabolites and enzymes of nitrogen metabolism in *Chlorella homosphaera* and *Scenedesmus quadricauda*.

## **MATERIALS AND METHODS**

### ***Study area for water samples collection:***

Water samples were collected from fish farming near Gamasa city-Dakahlia province - Egypt. Water surface samples were collected during March and April 2008. The samples were directly transported to the laboratories of Botany Department, Mansoura University for analysis. Water sampling followed USEPA (1985).

### ***Isolation and purification of planktonic microalgae :***

Composite freshwater samples were centrifuged at 4000 rpm for 10 minutes. The supernatant was decanted and the planktonic pellets were collected into sterile test tubes containing sterilized MBL medium (Nichols, 1973) as general freshwater phytoplankton growth medium. Isolation procedure of Anderson (2005) was used in the present investigation for isolation of *Chlorella* and *Scenedesmus isolates*.

The Fresh biomass was used to isolate microalgae following the streak plate method. This method serves to isolate an algal unit and may produce an axenic culture. The streak plate method involves: Petri dishes containing MBL medium (pH 7.2) solidified with 1 – 1.5 % agar were prepared. The solid medium should be half or two thirds the depth of the dish. One to two drops of algal biomass were placed near the periphery of the agar. Using a septic technique, parallel streaks of the suspension were made on the agar using a flame sterilized wire loop. The plate was covered and incubated for 48 days at  $24 \pm 2$  °C and continuous light of 2.789 w/m<sup>2</sup>. Using a stereomicroscope, algal colonies that are free of other organisms were spotted and used for further isolation using a fine capillary pipette or fine wire needle. Streaking procedure was repeated with algal units from single colony on agar nutrient medium. Finally the pure monoalgal cells were transferred to liquid or solid medium. The purity of the culture was ensured by regular microscopic examination. This method resulted in isolation of two different species of *Chlorella* and *Scenedesmus*.

**Identification of *Chlorella* and *Scenedesmus* isolates based on morphological characteristics**

Microscopic examination indicated the monoculture nature of both *Chlorella* and *Scenedesmus* isolates. Identification was based on gross morphological characteristics (Komaréck and Fott, 1983). The two isolates are identified as *Chlorella homosphaera* (Skujja) and *Scenedesmus quadricauda* (Turp.) de Brébisson.

**Nomenclature and taxonomy of the test *Scenedesmus* and *Chlorella* species**

The most recent information on algal taxonomy are posted at the web page AlgaeBase (<http://www.algaebase.org/>) that providing up-to-date taxonomic information concerning classification of algal species. This web-page is continuously updated and revised in light of the newest results obtained by molecular genetic approaches such as DNA sequence comparisons. According to taxonomical information posted at AlgaeBase, the *Chlorella* and *Scenedesmus* species are classified as follow:

**1-*Scenedesmus quadricauda***

Empire : Eukaryota  
 Kingdom : Plantae  
 Phylum : Chlorophyta  
 Class : Chlorophyceae  
 Order : Sphaeropleales  
 Family : Scenedesmaceae  
 Subfamily : Scenedesmoidea  
 Genus: *Scenedesmus*  
 Species: *quadricauda*

**2) *Chlorella homosphaera***

Empire : Eukaryota  
 Kingdom : Plantae  
 Phylum : Chlorophyta  
 Class : Trebouxiophyceae  
 Order : Chlorellales  
 Family : Chlorellaceae  
 Genus: *Chlorella*  
 Species: *homosphaera*

**Estimation of photosynthetic pigments:**

The photosynthetic pigments chlorophyll a, b (Chl.a, Chl.b) and total carotenoids (Cart.) were extracted and determined using spectrophotometric method described by Einhelling method (1986).

**Determination of total free amino acids:**

Total free amino acid content was determined according to Misra *et al.* (1975).

**Determination of total carbohydrates :**

Total carbohydrate content was determined by a modified phenol-sulphuric acid method (Fales, 1951).

**Determination of the total lipid content :**

The total lipid content was determined by the methods of Holme and Hazel (1983).

**Determination of total phenols content:**

Extraction of total phenol content was carried out according to Malusà *et al.* (2006) and determined by the method of El-Shora and Ad El-Gawad (2014).

**Preparation of crude enzyme extract:**

Fresh weight (0.1 g) was suspended in 10 ml of 20 mM phosphate buffer (pH 7.0) and the mixture was blended in warren blender. The resulting homogenate was centrifuged at 5,000 rpm for 10 min. The resulting supernatant constitutes the crude enzyme extract which was used for subsequent analysis (El-Shora, 2002).

**Determination of nitrate reductase activity (E.C.1.7.1.1) :**

Nitrate reductase was assayed by the method of El-Shora and Ali (2011).

**Determination of nitrite reductase activity (E.C.1.7.2.1) :**

Nitrite reductase activity was measured following the method of (El-Shora and Ali, 2011).

**Determination of glutamine synthetase activity (E.C.6.3.1.2)**

The activity of glutamine synthetase was assayed by the method of El-Shora *et al.* (2008).

**Determination of urease activity (EC 3.5.1.5)**

This assay of urease activity was done according to El-Shora (2001).

**Determination of ammonia-N :**

The method used for estimation of ammonia-N was that of Delroy (1949) using Nessler's reagent as modified by Naguib (1964).

## RESULTS AND DISCUSSION

It is apparent from the obtained results (Fig. 1) that chlorophyll a content in *C. homosphaera* was about twice of that observed in case of *S. quadricauda* (Fig. 2) in the control samples (untreated). Treating both algae with humic acid it was observed that there was remarkable increase in Chl a in each alga.

However, it is noticed that the increase of chlorophyll a under treatment with humic acid for *S. quadricauda* was higher than the increase in chlorophyll a for *C. homosphaera*. It was found that Chl a content was 214 % of that recorded for the control sample in case of *S. quadricauda*, but the chlorophyll a content in the treated *C. homosphaera* was 140 % of the control sample.

It noticed that treatment of both algae with humic acid resulted in a remarkable increase in chlorophyll b content in both algae. The results of this experiment indicate that chlorophyll b content in *C. homosphaera* (Fig. 1)

was higher than that of *S. quadricauda* (Fig. 2).

However, it should be stressed that the increase in chlorophyll b content in treated *S. quadricauda* was higher than the increase in chlorophyll b for *C. homosphaera*. This was observed from the results which show chlorophyll b content of *S. quadricauda* was 225 % of that for the control sample whereas the chlorophyll b content in the treated *C. homosphaera* was 174 % of the control sample.

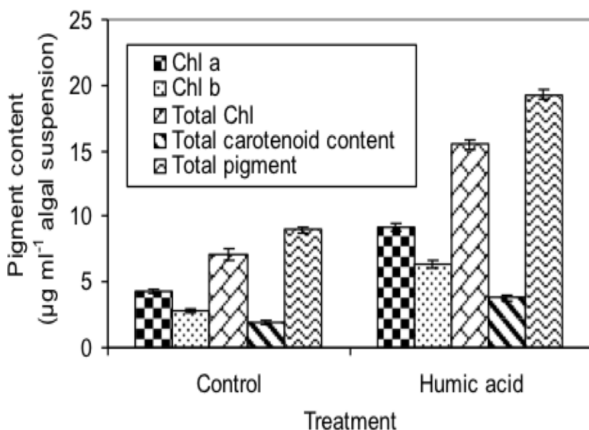
These results indicate an increase in the total chlorophyll content in both algae. However, the increase was most remarkable in case of *S. quadricauda* which expressed 218 % of that recorded in the control. In case of *C. homosphaera* the increase was 153 % of the control values.

These results revealed that carotenoid content in *C. homosphaera* was higher than that of *S. quadricauda*. In fact this phenomenon was observed for the control as well as for the treated algae. However, humic acid treatment resulted in comparable increase in carotenoid

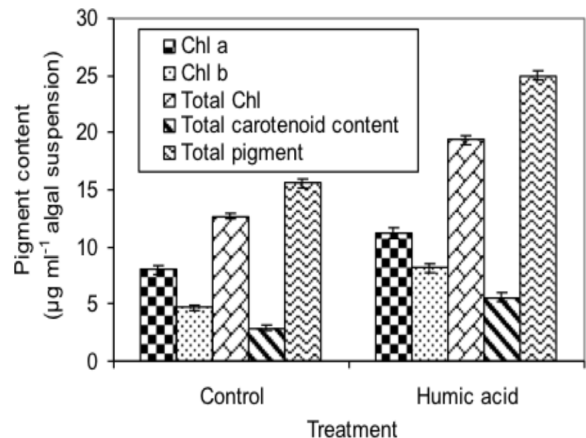
content in both algae and this was shown through the calculated percentage of carotenoid content related to the control samples for both algae. The percentages related to the control samples were 200 % and 193 % for *S. quadricauda* and *C. homosphaera*, respectively.

The above results are in harmony with those of Udayamali and Lokuhewage (2011) who reported that chlorophyll a was increased after treatment of *Ulothrix zonata* with humic substances. In intensive cultures, lower humic acid concentrations increased chlorophyll and carotenoid content (Pouneva, 2005).

The important role of biostimulants on enhancing the algal chlorophyll might be attributed to their action on increasing the availability of water and minerals. The high chlorophyll content might have resulted from enhanced algal growth (Mady, 2009). Changes in the chlorophyll, is another important growth parameter. Chlorophylls and carotenoids contents were increased in response to humic acid in both algae under the present investigation.



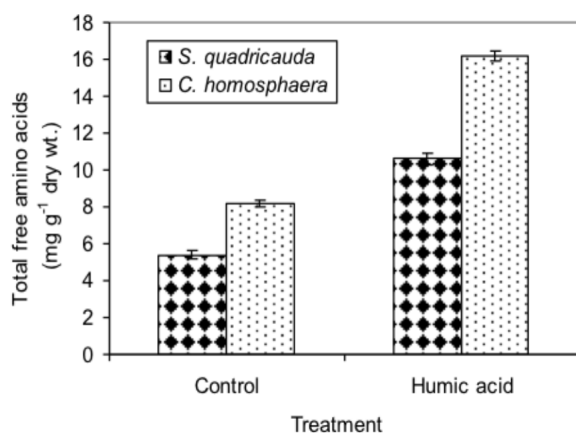
**Fig. (1)** : Effect of humic acid on pigment content of *S. quadricauda*.



**Fig. (2)** : Effect of humic acid on pigment content of *C. homosphaera*.

The total free amino acids increased in both algae (Fig. 3). The increase in the amino acids contents could be attributed to the fact that increasing photosynthesis by humic acid resulted in an increase in carbohydrates and keto acids contents, consequently amino acids could be synthesized through the transamination reaction between keto acids and amino acids. Also, nitrogen bound to humic substances may directly or indirectly stimulate algal growth (Gagnon *et al.*, 2005) and synthesis of amino acids.

Another possibility that humic acid treatment might induce the uptake of nutrients (Bohme and Thi, 1997), particularly nitrogen and thus stimulated nitrate reductase, nitrite reductase and glutamine synthetase leading to synthesizing more amino acids and conse-



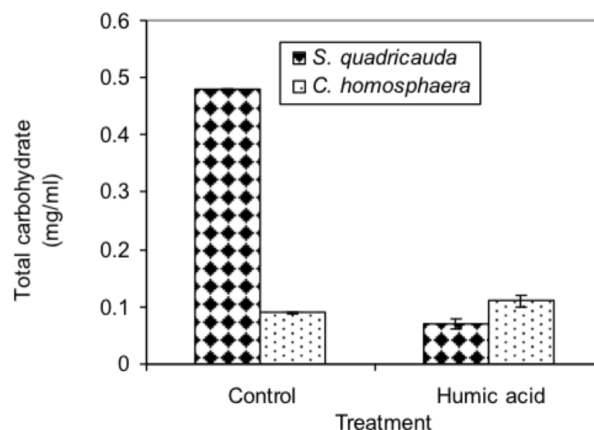
**Fig. (3)** : Effect of humic acid on total free amino acid content of *S. quadricauda* and *C. homosphaera*

The total lipid content increased in both algae after treatment with humic acid (Fig. 5). The lipid and carbohydrate content induced by humic acid. It was concluded that humic acid might be helpful in the improvement of algal viability and growth (Pouneva, 2005).

quently induced the incorporation of the amino acids for the synthesis of protein.

The accumulation of free amino acids may supply energy for growth and might function a scavenger or act as osmolytes and thus help the algal cell to grow well (Snaker *et al.*, 2007). It has been reported that amino acids as compatible osmolytes accumulate to balance the ions of Cl<sup>-</sup> and Na<sup>+</sup> that might accumulate in the cell (Yassen and Abu-Basal, 2008).

The results in the present work showed that humic acid resulted in the increase of total carbohydrate content (Fig. 4). Gill *et al.* (2002) reported that soluble sugars play an important role in the osmotic regulation of cells during reproduction.



**Fig. (4)** : Effect of humic acid on total carbohydrate content of *S. quadricauda* and *C. homosphaera*.

The treatment of both algae with humic acid in the present study resulted in an increase in the total phenols (Fig. 6). The term phenolic compound embraces a wide range of substances. Phenols comprise an aromatic ring, bearing one or more hydroxyl substanc-

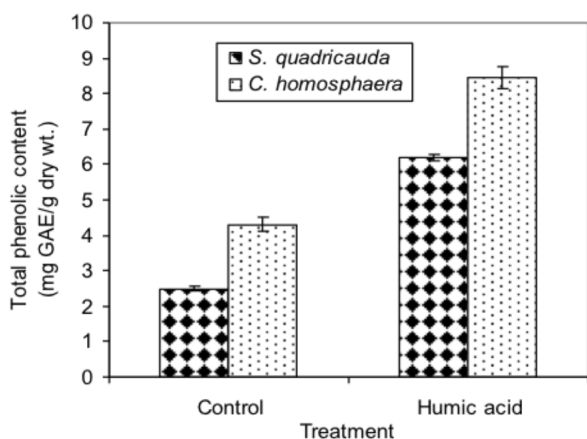
es, and range from simple phenolic molecules to highly polymerized compounds (Bravo, 1989). Also, the increase of phenolic content under treatment with humic acid is important for the cells to scavenge any free radical formed and thus protect the cells from oxidative damage during the growth.

Treatment of both algae with humic acid resulted in the increase of the activities of NR, NiR, GS and urease. In support, Shahryari *et al.* (2013) reported that potassium humate increased NR activity. The increase in the activities of the enzymes of nitrogen metabolism possibly may be due to increasing the uptake of nitrogen by humic acid. Humic acid concentrations increased  $\alpha$ -esterase and glutamate dehydrogenase activities (Pouneva, 2005).

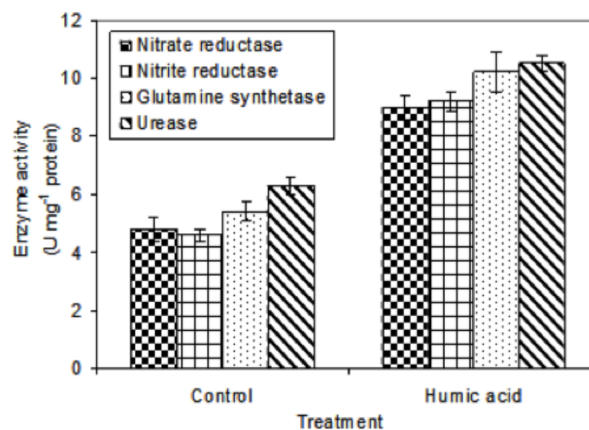
The photochemical breakdown of humic substances provides a source of substrates for microorganisms by at least four pathways

(Kieber, 2000): (i) by increasing the bioavailability of molecules that are bound to humic substances (e.g., amino acids, carbohydrates, and aromatic compounds) (Jørgensen *et al.*, 1998); (ii) through photolytic formation of low-molecular-weight substrates such as organic acids, carbonyl compounds, and hydrocarbons (Bertilsson and Tranvik, 2000); (iii) by modifying the high molecular-weight fraction of humic substances rendering it more labile to microbial attack (Lindell *et al.*, 1995); and (iv) by increasing the pool of limiting inorganic nutrients (Bushaw-Newton and Moran, 1999).

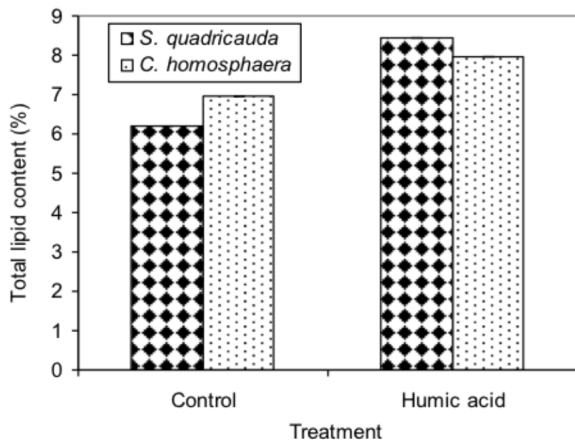
In conclusion, treatment of *Chlorella homosphaera* and *Scenedesmus quadricauda* with humic acid enhanced the pigments, metabolites as well as the enzymes of nitrogen metabolism. Therefore, it is suggested to apply humic acid in fish farming to increase the growth of algal species which are considered as feed for fish and it is an economic process.



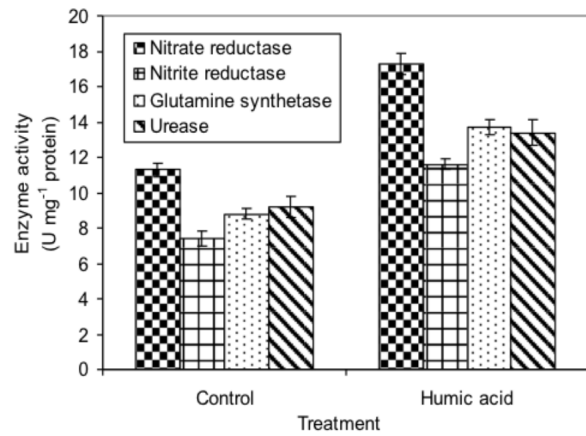
**Fig. (5)** : Effect of humic acid on the total lipid content of *S. quadricauda* and *C. homosphaera*.



**Fig. (6)** : Effect of humic acid on total phenolic content of *S. quadricauda* and *C. homosphaera*.



**Fig. (7)** : Effect of humic acid on enzymes activity of *S. quadricauda*.



**Fig. (8)** : Effect of humic acid on enzymes activity of *C. homosphaera*.

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## الملخص العربي

### تأثير الحامض الدبالي على الأصباغ ومواد الأيض وأنشطة إنزيمات أيض النيتروجين

حامد محمد الشورى<sup>١</sup> محمد إسماعيل عبد الحميد<sup>١</sup>  
محمد زين العابدين نصار<sup>٢</sup> سمير عبد القادر<sup>٢</sup>

<sup>١</sup> قسم النبات - كلية العلوم - جامعة المنصورة

<sup>٢</sup> المعهد القومي لعلوم البحار والمصايد

هدف هذا البحث إلى دراسة تأثير الحامض الدبالي بتركيزي ٣٠ ملليجرام لكل لتر و ٥٠ ملليجرام لكل لتر على الأصباغ والمواد الكربوهيدراتية والدهنية والفينولات في كل من كلوريل هوموسيفيرا وسنيديسمز كوادريكودا. وهدف أيضا البحث إلى دراسة تأثير الحامض الدبالي على نشاط إنزيمات أيض النيتروجين مثل ريدكتيز النترات و ريدكتيز النيتريت والأنزيم المصنع للجلوتامين وإنزيم اليوريز. بينت النتائج أن معاملة الطحلبين بالحامض الدبالي أدى إلى زيادة محتوى الأصباغ وكذلك المواد الأيضية السابق ذكرها في كل من الطحلبين. وكذلك أدت المعاملة إلى زيادة نشاط الإنزيمات المذكورة بدرجات متفاوتة في كل من الطحلبين.

JOESE 5

**INFLUENCE OF HUMIC ACID ON PIGMENTS, METABOLITES  
AND THE ACTIVITIES OF ENZYMES OF NITROGEN  
METABOLISM**

**\*Hamed M El-Shora, \*Mohammed I Abdelhamid, \*\*Mohamed Z. Nassar,  
and \*Samir Abdel-Kader**

*\*Botany Department, Faculty of Science, Mansoura University, Egypt*

*\*\*National Institute of Oceanography and Fisheries*

*shoraem@yahoo.com*

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