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Effects of Nitrate toxicity on free Proline accumulation, chlorophyll degradation and photosynthetic efficiency in the green alga *Chlorella vulgaris*

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Abstract: The effects of high nitrate concentrations on alterations in maximal photochemical efficiency of PSII ratio (Fv/Fm), chlorophyll content, chlorophyll degradation, growth rate and proline accumulation in *Chlorella vulgaris* Beyerinck [Beijerinck] were investigated in this study. The Fv/Fm ratio was observed in response to the increased nitrate concentration. The Fv/Fm ratio decreased in *C. vulgaris* following 44 h in 12.99 mM $NaNO_3$-enriched media. Experimental results showed that, growth rates and chlorophyll content declined by increasing nitrate concentration. The decrease in the ratio of chlorophyll a/b with enrichment of high nitrate concentration (6.5 mM and 12.99 mM $NaNO_3$) was also caused by a decrease in chlorophyll a and an increase in chlorophyll b concentration in *C. vulgaris* cultures. The results showed that 6.5 and 12.99 mM nitrate concentration increased proline content in treated cells, which suggests that nitrate stress lead to proline accumulation in *C. vulgaris.*

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1. INTRODUCTION

Nitrate occurs naturally in mineral sources and animal wastes, and anthropogenically as a by-product of agriculture and human wastes [1]. Ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) are the most common ionic (reactive) forms of dissolved inorganic nitrogen in aquatic ecosystems. $NaNO_3$ is commonly used as a nitrogen source in algae culture mediums. Nutrient concentrations play an important role in the growth of phytoplankton. The nitrogen sources that are considerably important for phytoplankton growth are nitrate and ammonium [2, 3]. It is a constituent element of amino acids and thus of protein, and nucleic acids (DNA and RNA). It typically makes up around 4% of the dry weight of plant matter, and around 3% of the human weight. It is one of the biggest component of animal waste, usually in the form of urea, uric acid, ammonium compounds and derivatives of these nitrogenous products, which are essential

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nutrients for all plants that are unable to fix atmospheric nitrogen. Much higher nitrate concentrations have been found in aquatic ecosystems that were strongly contaminated by agricultural and urban activities [4,5]. Enrichment of nitrogen and phosporus in aquatic enviroment can lead blooming of algae. Previous studies have also reported that nitrate could affect photosynthesis, growth and can cause cellular toxicity of phytoplankton [6, 7, 8].

Under different stress conditions, cells also tend to accumulate low intracellular molecular weight compounds in plants. Proline is such a compound that accumulates in higher plants and algae as a response to osmotic stress such as drought, high levels of salinity and osmolarity. In addition, air pollution causes an increase in the intracellular proline levels of a variety of higher plants [9]. Different roles have also been proposed for proline accumulation as an adaptive response; it has been suggested that proline may function as an osmoticum [10], a sink of energy and reducing power [11], a nitrogen-storage compound [12], a hydroxyl-radical scavenger [13] and a compatible solute that protects enzymes [14].

In higher plants, plant growth generally decreases when nitrogen supply exceeding 10 mM, a value considered on the threshold of toxicity for some species [15, 16]. Previous studies demonstrated that excessive nitrogen fertilization causes osmotic stress, in which reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) are produced [17]. ROS are highly toxic and can cause serious damages on lipid, protein and nucleic acid metabolisms and then inhibit plant and algae growth [18]. Although, there are some findings about high nitrate concentration lead to inhibition of phytoplankton growth, effects of high nitrate stress on chlorophyll content, chlorophyll degradation and free proline accumulation is still unexplored. In order to gain some insights on toxicity of nitrate in green algae, this study examined the effects of nitrate stress on growth rate, photosynthesis, and chlorophyll degradation. In addition, the objective of the present work is to determine the relationship between the nitrate toxicity and proline accumulation.

2. METHOD

2.1. Cultures and materials

Chlorella vulgaris was obtained from EGE-MACC culture collection, Ege University, Izmir, Turkey. Five flasks of 100 ml *C. vulgaris* were used for the experiment. *C. vulgaris* culture was grown photoautotrophiclly in Rudic Medium (RD) [19] at 31°C in under 12:12 dark:light illumination. Illumination provided by daylight fluorescence tubes at 20 μmol photons $m^{-2}s^{-1}$. Air was supplied to the culture. Continuous aeration was provided by bubbling air using a blower. Microalgae cells were harvested by centrifugation and transferred to a fresh medium, grown under the same conditions for a day for adaptation. Then the concentrations of 3.1 mM, 6.5 mM and 12.99 mM $NaNO_3$ were added to the nitrate stress groups, respectively. The cultures were sampled at 18^{th}, 24^{th} and 44^{th} hours. All the experiments were repeated three times.

2.2. Cell density

Absorbance at 663 nm was determined with UV-Vis spectrophotometer for all groups. Relative cell density demonstrated at different absorbances in Table 1. Specific growth rates (μ) were calculated using the equation $\mu=\ln(X_t/X_0)/t$, where X_0 is the initial cell density, X_t is the cell density after t hours.

2.3. Measurement of photosynthetic efficiency

Assay of photosystem II (PSII) activity was performed by fast chlorophyll fluorescence according to Strasser et al. [20], using Handy-Pea from Hansatech (King's Lynn, England). Five drops of cell suspension were pipetted on a piece of filter paper fixed in the clips of the

Handy-Pea and incubated in darkness for 15 min. Cells were kept moist and control showed the normal induction curves (Kautsky-effect) and values of the Fv/Fm ration of approximately 0.78. Each experiment was performed by five replicates.

2.4. Determination of pigment content

About 20 mg cells were extracted in 2 ml 90% acetone. Completely homogenized cells were subjected to quick centrifugation and supernatant was transferred to fresh tube. The absorbance of supernatant was taken at 664 and 647 nm in UV–Vis spectrophotometer. Chlorophyll is a light sensitive pigment hence light was avoided and all steps were carried out in dark. Each experiment was performed five times. Chlorophyll content was calculated using following formula [21]:

$$\text{Chla } (\mu g/ml): 11.93A_{664} - 1.93A_{647}$$

$$\text{Chlb } (\mu g/ml): 20.36A_{647} - 5.50A_{664}$$

2.5. Determination of Proline content

Proline content of the pellet was measured following the method of Bates et al. [22] with some modification of the extraction procedure according to Bačkor et al. [23]. Algae cells were permeabilized by 2 ml of DMSO at 40ºC for 1 h. Cell extracts were vortexed with glass beads 1 mm diameter for 30 s, and 2 ml of 3% sulfosalicylic acid was then added to each tube. After 10 min., extracts were separated from cell debris by centrifugation at 400 x g for 20 min. Two milliters of supernatant containing proline were pipette and subsequently treated with acid-ninhydrin at 90ºC for 1 h. The reaction was then terminated in an ice bath and the colored complex extracted in toluene. Absorbance was recorded at 520 nm. The standard curve for proline was prepared by dissolving proline in 3% sulfosalicylic acid to cover the concentration range 0.1-20 µg ml^{-1}. Each experiment was performed three times.

2.6. Statistical analysis

Statistical analysis was performed with one-way analysis of variance (ANOVA) or Student's t-test followed by *posthoc* Tukey test as appropriate (SPSS for Windows version 11.0)**.**

3. RESULTS

The effects of different nitrate concentrations on growth were shown in Table 1. Specific growth rates in 3.1 and 6.5 mM nitrate-enriched media for 24 h were approx. 1.7 and 1.2-fold that in 12.99 mM nitrate-enriched group, respectively. In addition, the specific growth rates and maximum cell densities of algae at 6.5 and 12.99 mM nitrate-enriched showed a significantly decrease ($p< 0.05$) in comparison with control groups after 44 h. The toxicity of nitrate at 12.99 mM was also evidently greater than at 6.5 mM (Table 1).

Photoinhibition measured as a permanent reduction in maximal PSII efficiency (Fv/Fm), increased with increasing level of nitrate in nutrient medium and treatment duration (Figure 1). The results of Fv/Fm measurements (Table 2) indicated a progressive photoinhibition by the values closed to 29.73% for 24 h in 6.5 mM nitrate-enriched media. After 44 h, a significantly decrease Fv/Fm (62.16 %) was measured in samples treatment with 12.99 mM nitrate ($p< 0.05$).

Table 1. Maximum cell densities and specific growth rates of the alga Chlorella vulgaris cultivated with a nutritional level of nitrate (control) and nitrate supplement of 3.1, 6.5, and 12.99 mM.

Duration (hours)	**Nitrate concentrations (mM)**	**Specific growth rates**	**Maximum cell densities (x10^5 cell mL^{-1})**
18	3.1	0.42	2.523±*112*
	6.5	0.32	2.132±*110*
	12.99	0.303	**1.931±*52***
24	3.1	0.41	2.706±*127*
	6.5	**0.3**	**1.918±*63***
	12.99	**0.25**	**1.219±*56***
44	3.1	0.39	2.534±*123*
	6.5	**0.28**	**1.472±64**
	12.99	**0.23**	**0.973**±68

Table 2. The Fv/Fm ratio of the alga Chlorella vulgaris cultivated with a nutritional level of nitrate (control) and nitrate supplement of 3.1, 6.5, and 12.99 mM.

Groups		Fv/Fm			Photoinhibition (%)		
	n	18 h X±SD	24 h X±SD	44 h X±SD	18 h X±SD	24 h X±SD	44 h X±SD
Control	5	0.74±0.03	0.73±0.16	0.74±0.14	1.34±0.05	2.67±0.026	1.34±0.02
3.1 mM $NaNO_3$	5	0.72±0.22	0.72±0.04	0.73±0.03	2.71±0.03	2.7±0.34	1.36±0.04
6.5 mM $NaNO_3$	5	0.61±0.32	0.6±0.18	**0.52±0.08^a**	**17.57±1.2^a**	**18.92±1.4^a**	**29.73±1.10^a**
12.99 mM $NaNO_3$	5	**0.56±0.03^a**	**0.45±0.12^a**	**0.28±0.23^b**	**24.33±0.07^a**	**39.19±0.012^{ab}**	**62.16±1.03^{ab}**

On nutritional *C. vulgaris* media and 3.1 mM nitrate supplemented media, the pigment levels were not significantly different (Table 3), but they did differ between groups in the presence of different nitrate concentration. There are significant ($p<0.05$) differences determined on Chl*a*, Chl*b* and Chl*a/b* levels between control group and nitrate treated groups, with the only exception of the 3.1 mM nitrate treated group (Table 3 and 4). It was observed that chlorophyll a content, and chlorophyll a/b ratio of the 6.5 mM and 12.99 mM nitrate-enriched media treated *C. vulgaris* for 44 h were reduced by 43.7%, 68.65% and 55.37%, 82.51% respectively.

Table 3. Chlorophyll a (Chla) (mg g^{-1} dw cell mass) and Chlorophyll b (Chlb) (mg g^{-1} dw cell mass) contents of the alga Chlorella vulgaris cultivated with a nutritional level of nitrate (control) and nitrate supplement of 3.1, 6.5, and 12.99 mM.

Groups		Chl*a*			Chlb		
	n	*18 h X±SD*	24 h X±SD	44 h X±SD	*18 h X±SD*	24 h X±SD	44 h X±SD
Control	*5*	*6.02±0.05*	*5.68±0.21*	*6.14±0.03*	*1.86±0.23*	*1.84±0.4*	*2,02±0.12*
3.1 mM $NaNO_3$	*5*	5.92±0.42	5.19±0.29	5.62±0.02	1.98±0.01	1.82±0.17	**1.69±0.19^a**
6.5 mM $NaNO_3$	*5*	**4.18±0.04^a**	**3.88±0.09^a**	**3.457±0.03^b**	**2.14±0.05^a**	**2.73±0.05^b**	**3.582±0.07^a**
12.99 mM $NaNO_3$	*5*	**3.01±0.2^b**	**3.20±0.04^a**	**2.74±0.24^a**	**3.09±0.1^a**	**3.61±0.06^b**	**4.95±0.04^b**

Table 4. Chlorophyll a /Chlorophyll b (Chla/b) ratio of the alga Chlorella vulgaris cultivated with a nutritional level of nitrate (control) and nitrate supplement of 3.1, 6.5, and 12.99 mM.

Chl*a/b*	*n*	18 h X±SD	24 h X±SD	44 h X±SD
Control	*5*	*3.23±0.05*	3,08±*0.14*	*3.03±0.04*
03.1 mM $NaNO_3$	*5*	2.97±0.24	2,83±0.34	3.32±0.23
6.5 mM $NaNO_3$	*5*	**1.95±0.12[a]**	**1.42±0.01[a]**	**0.95±0.01[a]**
12.99 mM $NaNO_3$	*5*	**0.97±0.06[a]**	**0.88±0.02[a]**	**0.53±0.13[a]**

Nitrogen-induced proline accumulation in the *C. vulgaris* cells are presented in Figure 1. The proline content of *C. vulgaris* arisen by increasing level of nitrate in nutrient medium and treatment duration. Proline accumulation in groups of enhanced media with 6.5 mM and 12.99 mM nitrate for 44 h were approx. 8 and 11 times greater than control group, respectively.

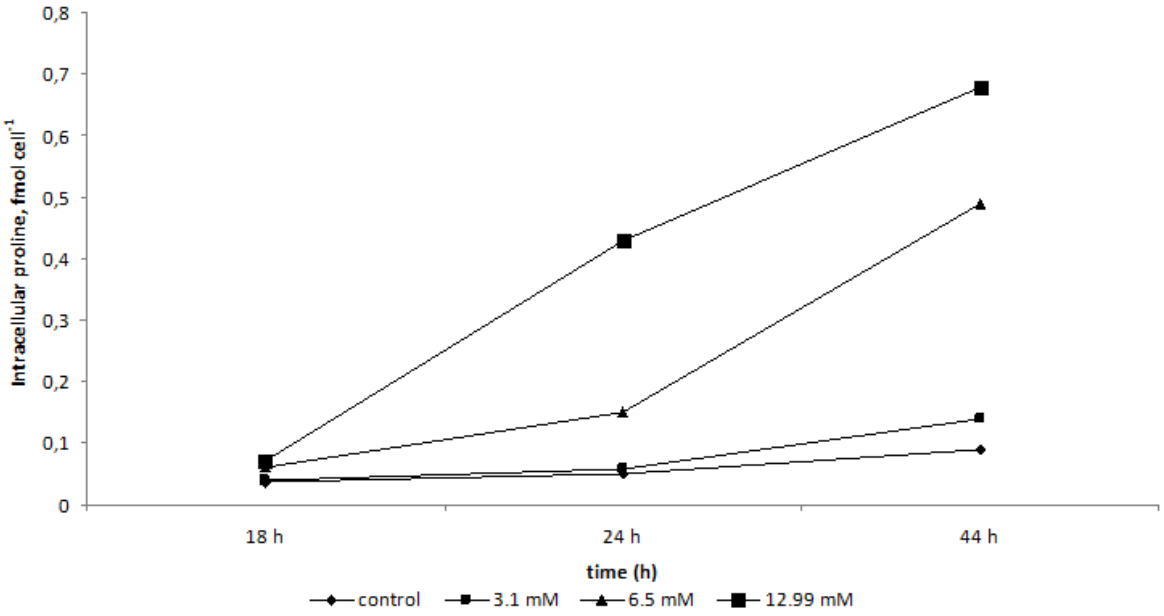

Figure 1. Proline accumulation levels according to nitrate concentrations at different time periods.

The results showed a positive correlation between proline accumulation and photoinhibition (Figure 2) also with chlorophyll degradation and growth inhibition (Figure 3).

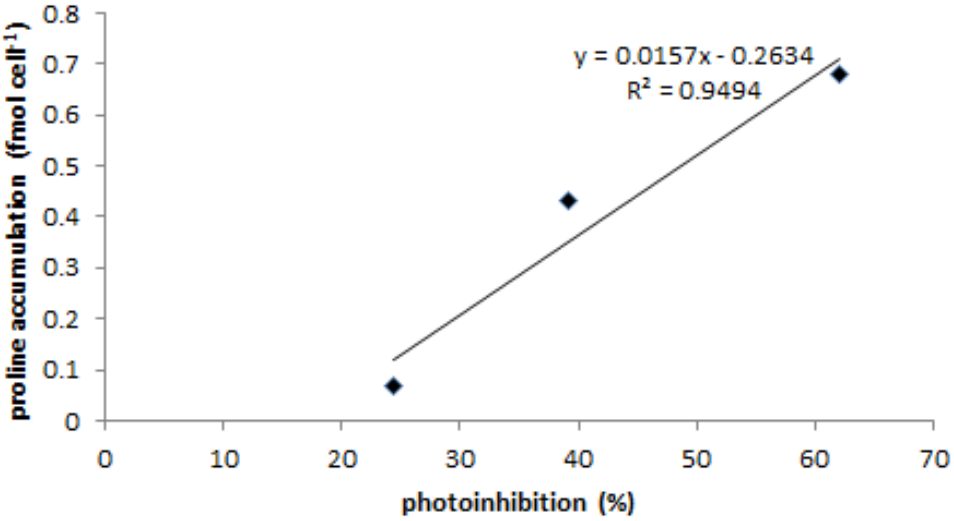

Figure 2. Relationship between proline accumulation and photoinhibition (%).

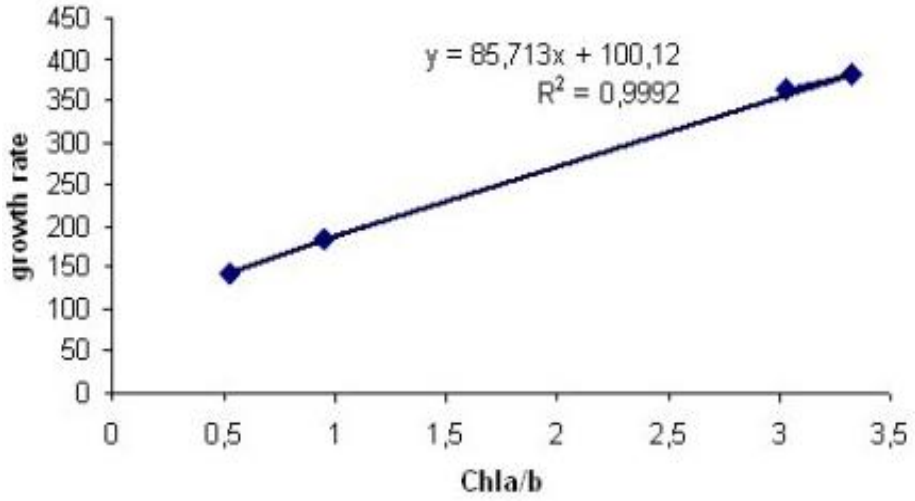

Figure 3. Relationship between chlorophyll degradation and growth rate of *Chlorella vulgaris*.

4. DISCUSSION

Nitrate is an important macronutrient and can serve as a good nitrogen source for phytoplankton. Menéndez [8] reported that moderate levels of fertilizing nitrate could increase the maximum rate of net photosynthesis in green alga *Chaetomorpha linum*. Touzet et al. [24] also found that the dinoflagellate *Alexandrium minutum* showed higher maximum cell densities with increasing nitrate concentrations. Similarly, Leong et al. [25] showed a positive correlation between the growth rates of *Alexandrium tamarense* and nitrate concentrations. However, Shi et al. [26] observed that there was a negative effect of excess nitrate on the growth of *A. tamarense*, and Hwang and Lu [27] also reported *A. minutum* exposed to excess nitrate had a low growth rate. These findings suggested that, although nitrate was a good nitrogen source, excess nitrate supply is harmful to phytoplankton and could depress growth, which is in agreement with the results of our present study.

Nitrate is converted to ammonium in two successive steps catalysed by nitrate reductase (NR) and nitrite reductase (NiR), and when nitrite formed by NR, the accumulation of

intracellular nitrite of alga will appear. Chen et al. [28] demonstrated that *Microcystis aeruginosa* grown under high nitrate concentrations showed a significant increase in NR activities, which is consistent with the results of Sivasankar and Oaks [29] and Crawford [30], where the activities of NR increased with nitrate raise. Chen et al. [28] observed a significant increase in intracellular nitrite content under high nitrate concentrations in *M. aeruginosa.* In general, excessive nitrate can be stored as non-toxic form of nitrogen in cells [31], but nitrite is an inorganic monovalent anion which affects the process of photosynthesis significantly. It is known that the inhibition of photosynthetic electron transport can [32, 33], change intracellular pH and damage cell membranes of algae [34, 35]. Therefore, it is possible that the increase of intracellular nitrite resulted in the decrease of growth and chlorophyll degradation at high nitrate concentrations [28]. In the present work, significant variations of the maximal potential quantum yield of PSII complex (Fv/Fm) were obtained in exposure to high dosages of nitrate in *C. vulgaris.* In particular fluorescence parameter results, a good indicator of physiological stress caused by heavy load of nitrate. *C. vulgaris* cells were cultivated in 6.5 mM and 12.99 mM nitrate-enriched media for 44 h, exhibited distinct photoinhibition at the end of the experiments. Photoinhibition rates close to 62% were recorded in 12.99 mM nitrate-enriched media after 44 h of exposure.

Chlorophyll is very sensitive to stress-initiated oxidative processes such as photo-oxidation [36-38]. Chlorophyll intermediate molecules are also potential chloroplast signals that could regulate photosynthetic gene expression, growth rates, and cell-death processes [39]. Our present results showed that decreasing growth rates are closely correlated with decreasing chlorophyll a/b ratio following to supplemental different nitrate concentration (Figure 2). Chlorophyll b is formed from chlorophyll a by the oxidation of the methyl group on ring II to the aldehyde [37] and the ratio of chlorophyll a/b is more sensitive to modification than chlorophyll a+b. In green plants, antenna size is determined by the amount of light-harvesting chlorophyll a/b protein complex that is associated with the photosystems [4]. Conversion of chlorophyll b to chlorophyll a not only impacts the chlorophyll a/b ratio but also is the first step of chlorophyll degradation. Our present results confirmed that supplemental nitrate causes a significantly increase in chlorophyll b and a concomitant decrease in chlorophyll a, consistent with accelerated conversion of one to the other (Table 4).

In response to environmental stress in plants, proline accumulation normally occurs in cytosol where it contributes substantially to the cytoplasmic osmotic adjustment [40-42]. Binzel et al. [41] showed that the glutamate pathway for proline biosynthesis is predominant under stress conditions such as high salinity and nitrogen limitation. In algae and higher plants, intracellular proline has been also regarded as an osmoprotectant that accumulates in relation to the level of intracellular potassium ions [9]. Wu et al. [43] showed that the enhanced intracellular proline accumulation during the exposing to heavy metals related to a mechanism of protection against osmotic change.

Despite the presence of a strong correlation between stress tolerance and accumulation of proline in higher plants, this relationship may not be universal. For example, Lutts et al. [44] demonstrated that the accumulation of proline in rice leaf may be a symptom of salt injury rather than an indication of salt tolerance. De-Lacerda et al. [45] assessed the proline accumulation and distribution during shoot and leaf development in two sorghum genotypes. As a result they suggested that proline accumulation was a reaction to salt stress, not a plant response associated with tolerance. Similarly, Sánchez et al. [46] also found that the metabolic response of proline under nitrogen toxicity is reflected primarily in seeds of *Phaseolus vulgaris*. In the present work, proline accumulation in 12.99 mM nitrate-enriched media was significantly

higher than the 3.1 mM and 6.5 mM nitrate-enriched media for 24 h and 44 h. The present observations showed a positive relationship between nitrogen toxicity and proline accumulation.

In conclusion, the present work demonstrates that high nitrate stress decreases the growth rate and maximal photosystem II efficiency (Fv/Fm) in *C.* while chlorophyll degradation and proline accumulation increased according to treatment duration. Photoinhibition (%) data demonstrated that there is a possitive correlation between nitrogen toxicity and proline accumulation (Figure 2). In addition, our chlorophyll degradation and growth inhibition results are supporting this hypothesis (Table 1 and 4, Figure 3). These results define, proline accumulation as a bioindicator of nitrogen toxicity in the cells of *C. vulgaris.*

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