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THE USE OF A BIOLOGICAL RESPONSE TO DIFFERENT CONCENTRATIONS OF NITRATES AND PHOSPHATES IN FRESHWATER ALGAE *CHLORELLA VULGARIS* AS AN INDICATOR OF ENVIRONMENTAL IMPACT ASSESSMENT

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Abstract

The current study deal with biological response to fresh water green algae *Chlorella vulgaris*, due to its exposure to some environmental effects, which included different concentrations of nutrients (nitrates and phosphates) and the possibility of using these biological indicators as a tool to assess the potential environmental impact. *Chlorella vulgaris* was treated with concentrations of nitrates (10, 15, 20) mg /l and phosphate (0.1, 0.5, 1) and the experiments lasted for 14 days during which many physiological and biochemical parameters were this included growth curve, doubling time, photosynthesis pigments (chlorophyll-a and carotenoids), as well as measuring variation in total protein and carbohydrates, as well as studying the effect of previous factors on some enzymatic parameters such as Catalase, SOD and ROS and the results were recorded in (1 days, 7 days, 14 days). Measured in the algae under test, the maximum growth rate was 1.365 during 10th day with 20mg/l NO_3 treatment, while the minimum was 0.220 during 2nd day with 10mg/l and the maximum doubling time was 1.370 during 2nd day with 10mg/l NO_3 treatment, while the minimum was 0.220 during 10th day with 20mg/l. The maximum chlorophyll-a was 0.438 µg/ml during 14th day with 20mg/l NO_3 treatment while the minimum was 0.122 µg/ml during 7th day with 0.5mg/l, the maximum carotenoids was 0.051 µg/ml during 14th day with 20mg/l NO_3 treatment, while the minimum was 0.007 µg/ml during 14th day with 0.5 mg/l.

Key words: environmental impact; ions concentrations; biological response.

Introduction

Algae are autotrophic organisms like plants that belong to the kingdom of protists, most are single-celled, but some are large in size and multicellular (Graneli, 1984).

Algae differ from primates in that they are phototrophic, in which the chlorophyll synthesis is identical with all the more complex plants beginning with mosses and are thalosic creatures containing chlorophyll, it lives in environments with varying levels of humidity between water and dry desert sands, salinity levels between rain and sea water and temperatures between ice and hot springs (Oheocha, 1962).

Algae plays an important role in maintaining the balance of all ecosystems, It is the primary source of food for the rest of the neighborhoods, directly or indirectly and a major guarantee to purify the system of carbon dioxide and replace it with the oxygen, the role of algae is in purifying polluted environments from many types of pollutants by breaking them into non-harmful compounds in cases of biological or petroleum contamination or by absorbing them from the medium in cases of heavy metal contamination and by doing so they displace a huge amount of pollutants in the interest of the lives of many of the neighborhoods they live with them, also it used as indicators important to certain types of radioactive contamination, thermal, biological (Strack *et al.,* 1980).

Nitrogen and phosphorous compounds are among the essential elements that algae and aquatic plants need for growth as well as potassium, calcium, magnesium and other elements as phosphorus plays an important role in controlling the metabolic activities of the cell and producing energy, it is an essential component of nucleic acids as well as the importance of phosphorus in the breathing process and the source of phosphorus in wastewater and

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natural water (Al-Hussieny *et al.*, 2014).

Environmental assessment is the best method to clear the environmental state and describe the probable significant impact of external stress and use the biological tools to assist in the achievement of sustainable use of resources (Hiwase and Hajare, 2017).

Environmental assessment involve evaluating many environmental consequences such as terms of number, site of products, ecological capacity and biomass production (Gunderson, 2008).

Material and Methods

Algal strains, Preparation and Sterilization of Media

The *C. vulgaris* was identified by microscopic observation (Maulood *et al.*, 2013) and incubated under controlled conditions of light intensity 286 µE/m²/s, light/dark period 16:8 hours and temperature 25±2°C (Chia *et al.*, 2013).

All equipments and media were sterilize in autoclave at 121°C, 1.5 j for 15 min. Modified Chu-10 was used for the algal growth (Kassim *et al.,* 1999). The stocks were prepared for all macro and micro elements were dissolving weight of the salt as it is clearly demonstrated in table 1 in one liter of distilled water, 2.5 ml was taken from each stock solution and completed up to one liter of distilled water, then sterilized with autoclave, except stock solution (K_2HPO_4), which sterilized alone and added finally to get one liter of Chu-10 and its pH was set on 6.4 after the sterilization using (0.01N) of sodium hydroxide or hydrochloric acid.

Table 1: The components and concentration of modified Chu-10 medium and the concentration of each component (Kassim *et al.,* 1999).

Number of stock solution	Chemical formula of each salt	Concentration g\l
1	$MgSo_4.7H_2O$	10
2	K_2HPO_4	4
3	$NaNo_3CaCl_2$	816
4	$FeCl_3$	0.32
5	EDTA-Na	4
6	NaCl	30
7	Na_2Co_3	8
Trace metals mix A6	As shown below	
1	H_3BO_3	0.288
2	$MnCl_2.4H_2O$	0.02
3	$ZnSO_4.7H_2O$	0.224
4	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.028
5	$CuSO_4.5H_2O$	0.08
6	$CoCl_2.6H_2O$	0.004
7	Distilled water	1 L

10 ml of culture algal is taken in flask containing 100 ml of Chu-10 medium and grown for 15 days. This culture transported into 1000 ml of media and incubated for 14 days (Photo 1). Biomass algal increase in glass pools 5L (Tredici, 2004).

The algal *Chlorella vulgaris* (100 ml) was cultured in (1 liter) Chu-10 medium and left for at least two weeks before starting experiment under constant laboratory conditions.

The culture medium of *C.vulgaris* are exposed of two types of nutrients are represented by the potassium phosphate compound with a concentration (0.1, 0.5, 1 mg/l) as well as the sodium nitrate compound with a concentration (10,15,20 mg/l) and using culture media with algae as control without adding anything is examined the changes that occur daily on algae for a period of (14) days and are incubated to know the changes in the growth rate (k), doubling time (G) and then measure chlorophyll A, carotenoids, carbohydrates, Protein in (1^{st}, 7^{th}, 14^{th}) days.

Determination of Growth Rate and Doubling Time

To calculate the growth constant K and according to the following equation (Huang *et al.*, 2002a):

$$K = (\log OD_t - \log OD_0) \times 3.332 / t$$

K: growth rate

t: time

OD_0: optical density at the beginning of the experiment (zero time).

OD_t: optical density after (t) day.

As for the generation time of multiplication of G, it is calculated from the following equation (Huang *et al.*, 2002b):

$$G = 0.301 / K$$

G: doubling time

Estimation of Chlorophyll and Carotenoid

The amount of chlorophyll-a and carotenoids is estimated based on a method (Alam *et al.,* 2008) by taking 2 ml of the sample and discarding it at a speed of 12500

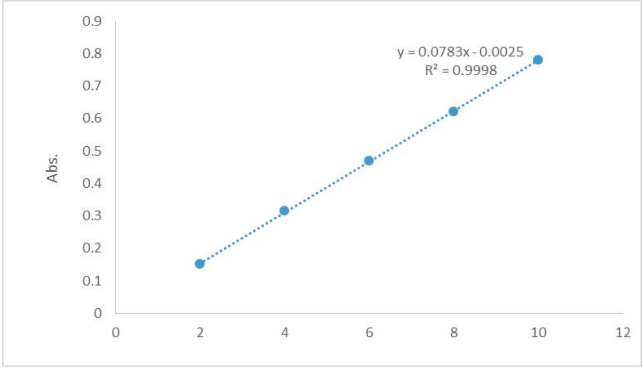

Fig. 1: Stander curve of glucose.

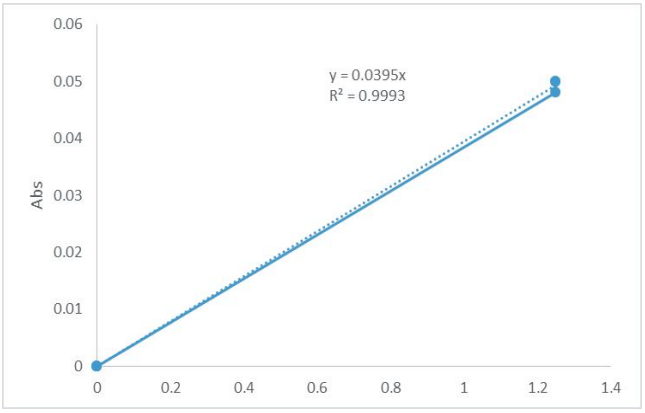

Fig. 2: Stander curve of albumin.

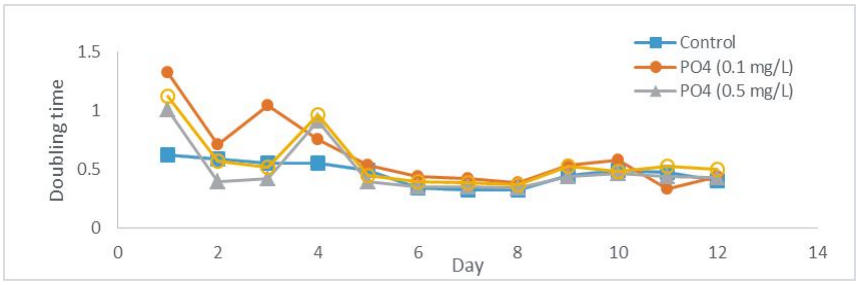

Fig. 3: Growth rate of different concentration of NO_3 treatment.

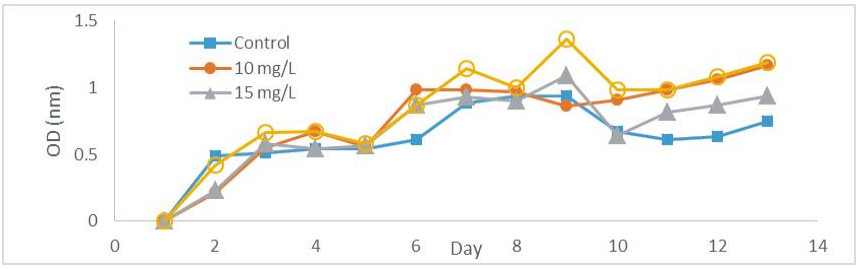

Fig. 4: Growth rate of different concentration of PO_4 treatment.

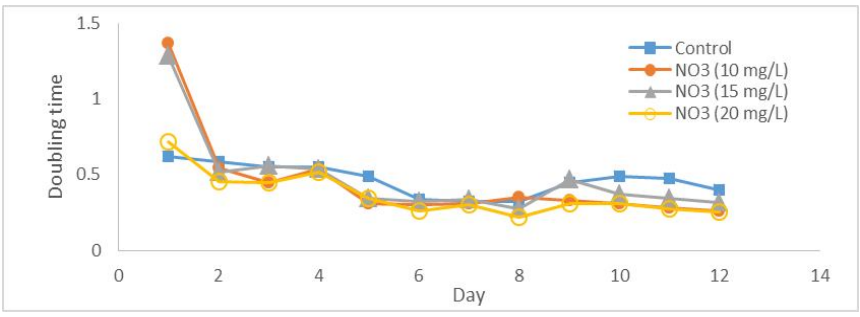

Fig. 5: Doubling time of different concentration of NO_3 treatment.

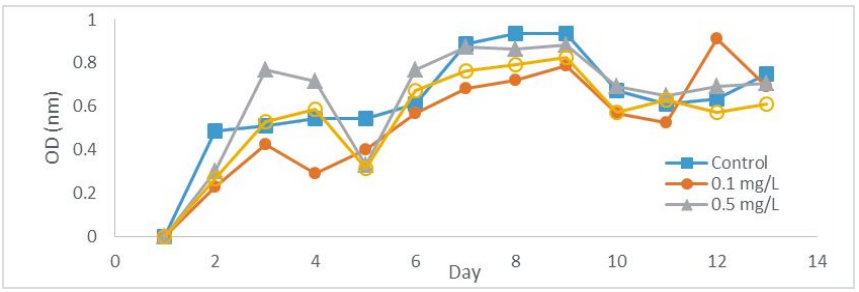

Fig. 6: Doubling time of different concentration of PO_4 treatment.

r/min for 5 minutes and taking the precipitate from algae and adding 2 ml methanol (90%) and placed in a water bath 64 degrees for 5 minutes after incubating for 20 hours in a place Darkness at a degree of 20 degrees and discarded at 12500 r/ min for 5 minutes. The filtrate is taken and measured at three different wavelengths 470, 652 and 666 nanometers. The chlorophyll and carotenoids are calculated from the following equations :

µg Chlorophyll / ml medium = (16.29 × A665) - (8.54 × A652) (Porra *et al.*,1989).

µg total carotenoids / ml medium = [(1000 × A470 - 44.76 × A666) / 221] (Kaczmar, 2004).

Estimation of Carbohydrates

2 ml of the sample is taken and dried aerobically after washing with Phosphate-buffer solution and breaking it with the sonicare and diluted to 5ml with distilled water and take 1 ml of it and add to it 5 ml of sulfuric acid (96%) and 1 ml of phenol (5%) and wait 10 minutes with continuous stirring is then placed in a water bath (30-35°C) for 10 minutes, then measured along 490 nm and compared with the standard curve of glucose prepared from dissolving 100 mg of glucose in 100 ml distilled water (Herbert, 1971).

Estimation of total protein

The total proteins were determined according to the LOWRY method modified by (López *et al.*, 2010) by taking 0.5 ml of the previously prepared extract and adding 2 ml of Biuret solution after mixing it with a preheater that was heated to 30 degrees for half an hour and then measured along 555 nanometers and Compare with the standard solution depending on the Bovin serum albumin protein at concentrations (0-0.1) ml, which is prepared by dissolving 0.1 g of Bovin with 100 ml of puffer solution, so that the concentration is 100 µg / l.

Results and Dissection

The maximum growth rate was 1.365 during 10th day with 20mg/L NO_3 treatment, while the minimum was 0.220 during 2nd day with 10mg/L (Fig. 3) and the maximum value of growth rate was 0.912 during 13th day with 0.1 mg/L of

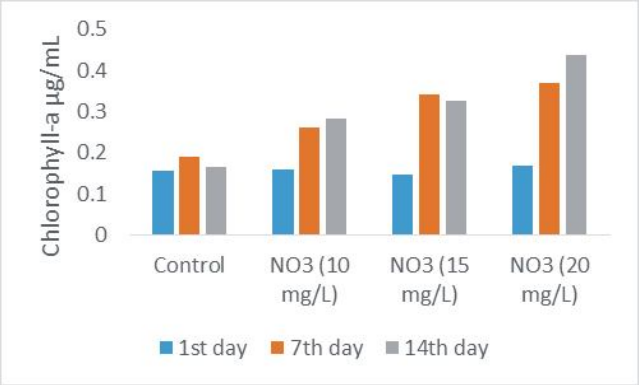

Fig. 7: Chlorophyll-a concentration in different concentration of NO_3 treatment.

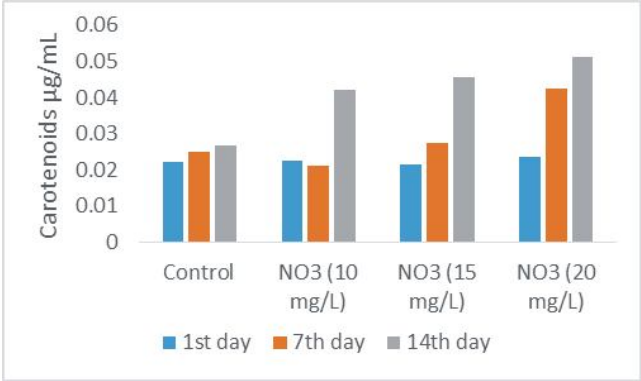

Fig. 9: Carotenoids concentration in different concentration of NO_3 treatment.

PO_4 treatment, while the minimum was 0.227 during 2nd day with 0.1mg/L (Fig. 4).

Nitrogen is required by a plant that enters into the vacuole and then is stored in large quantities as NO_3 and reduced by a nitrate-reductase enzyme. Finally, NO_2 is converted into ammonia (NH_3) in the chloroplast. Plants have large quantities of nitrogen compounds than any other nutrient type (Crawford, 1995). Moreover, the balance of nutrients plays an significant role in algae and biofuel production. The results showed that the maximum growth rate was 20mg /L NO_3 during the 10th day, while the minimum was 10mg /L during the 2nd day and the growth rate was also significantly increased with days and increased nitrate concentrations ($p<0.05$). These results have been agreed with most of the previous studies (e.g. Al-Shahiry *et al*., 2008; Paes *et al*., 2016; Altin *et al*., 2018). They also found that the optical density increased with elevated NO_3 values, the phosphate treatments the growth rates were increased significantly with day and with increasing of phosphate concentration ($p<0.05$), this results was agreed with (Baiee and Salman, 2016); (Chia *et al.,* 2017) and (Fu *et al.,* 2019) since it improves rapid microalgae growth and maturation (Cloern, 2001).

The maximum doubling time was 1.370 during 2nd day with 10mg/L NO_3 treatment, while the minimum was 0.220 during 10th day with 20mg/L (Fig. 5) the maximum value of doubling time was 1.327 during 2nd day with 0.1 mg/L of PO_4 treatment, while the minimum was 0.330 during 13th day with 0.1 mg/L (Fig. 6).

As shown in the results of the current study, doubling time was reduced on an orderly basis with days to all of treatment, the study showed that there were negative correlation between K and D and that the low doubling time corresponds to the high specific rate of growth (Liu *et al.,* 2011).

Chlorophyll-a: The maximum chlorophyll-a was 0.438 µg/ml during 14th day with 20mg/L NO_3 treatment, while the minimum was 0.146 µg/ml during 1st day with 15mg/L (Fig. 7).

But The maximum value of chlorophyll-a was 0.183 µg/ml during 7th day with 0.5 and 1 mg/L of PO_4 treatment, while the minimum was 0.122 µg/ml during 14th day with 0.5mg/L (Fig. 8) the maximum carotenoids was 0.051 µg/ml during 14th day with 20mg/l NO_3 treatment, while the minimum was 0.021 µg/ml during 7th day with 10mg/l (Fig. 9).

But the maximum value of carotenoids was 0.027 µg/ml during 7th day with 1 mg/l of PO_4 treatment, while

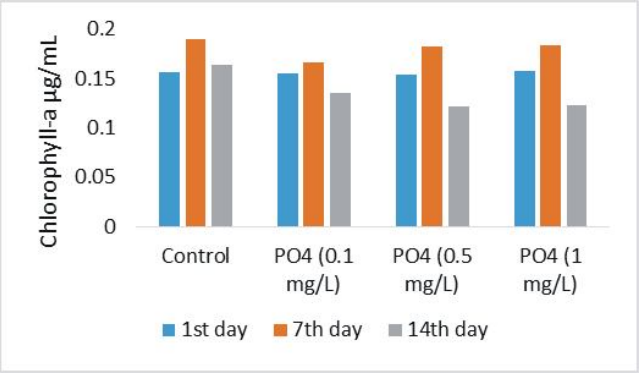

Fig. 8: Chlorophyll-a concentration in different concentration of PO_4 treatment.

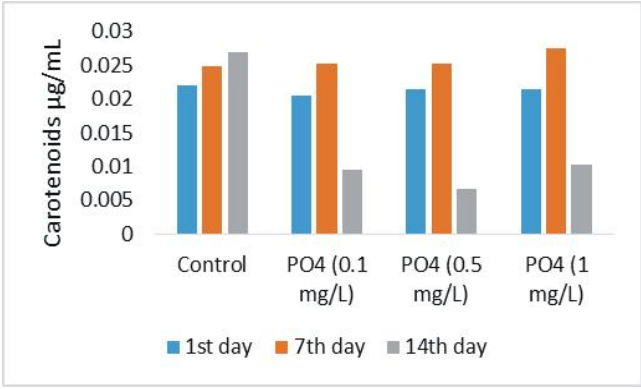

Fig. 10: Carotenoids concentration in different concentration of PO_4 treatment.

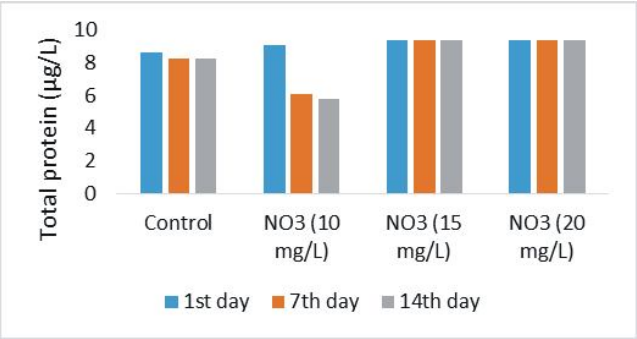

Fig. 11: Total protein concentration in different concentration of NO_3 treatment .

the minimum was 0.007 µg/ml during 14th day with 0.5 mg/L (Fig. 10).

The results showed that chlorophyll-a and carotenoids were increased significantly with increasing NO_3 concentrations ($p<0.05$) and correlated positively with days of treatments ($r= 0.87$, $p<0.05$), also the highest was recorded at 14th day, this results were conducted with previous studies e.g. (Ruiz *et al.,* 2011); (Chia *et al.,* 2013; Fu *et al.,* 2019). But, with PO_4 the concentrations of cl-a and carotenoids were decreased in 14th day in all treatments compared with control ($p<0.05$), while the studied pigments were increased significantly in 7th day ($p<0.05$) in all of treatments and control because that day represent an exponential phase and that agree with (Fogg and Thake, 1987). The results showed that ch-a and carotenoids were increased significantly with increasing NO_3 concentrations ($p<0.05$) and correlated positively with days of treatments ($r=0.87$, $p<0.05$), also the highest was recorded at 14th day, this results were conducted with previous studies e.g. (Ruiz *et al.,* 2011); (Chia *et al.,* 2013; Fu *et al.,* 2019). But, with PO_4 the concentrations of cl-a and carotenoids were decreased in 14th day in all treatments compared with control ($p<0.05$), while the studied pigments were increased significantly in 7th day ($p<0.05$) in all of treatments and control because that day represent an exponential phase and that agree with (Fogg and Thake, 1987).

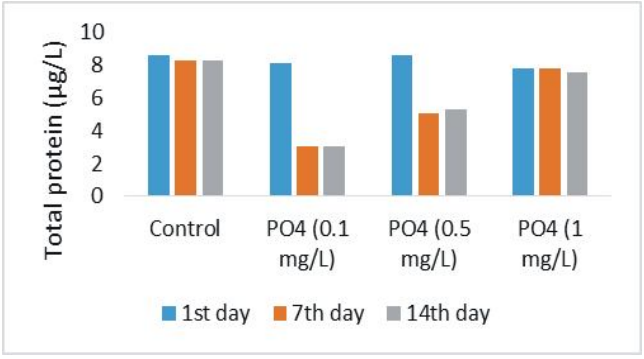

Fig. 12: Total protein concentration in different concentration of PO_4 treatment.

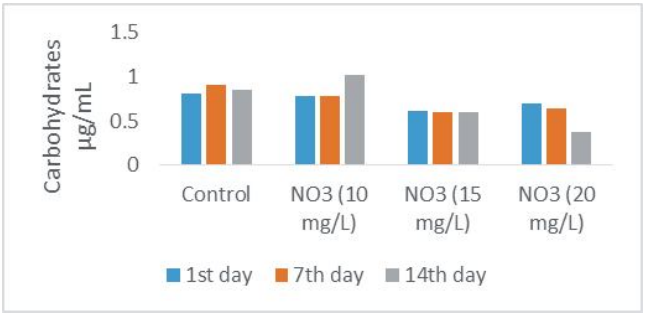

Fig. 13: Carbohydrates concentration in different concentration of NO_3 treatment.

The maximum total protein was 9.367 µg/L during all the time of experment day with 15 and 20 mg/L NO_3 treatment, while the minimum was 5.823 µg/L during 14th day with 10mg/L (Fig. 11).

But the maximum value of protein was 8.608 µg/L during 1st day with 0.5 mg/L of PO_4 treatment, while the minimum was 3.038 µg/L during 7th and 14th days with 0.1 mg/L (Fig. 12).

In *C. vulgaris*, the increasing of NO_3 concentrations mostly leads to increasing of proteins production (Al-Shahiry *et al*., 2008) this was agreed with our results that we showed that the NO_3 affected protein production positively ($p<0.05$). According to PO_4 treatments, the results showed that there were significant decline of protein contained ($p<0.05$) in 7th and 14th days of 0.1 mg/L and 0.5 mg/L treatments compared with control and 1st day of experiment. While in the highest concentration of PO_4, there were no significant differences compared with time and control and that was disagreed with (Baiee and Salman, 2016) that protein content elevated significantly, that may be because other conditions than PO_4 affects like pH, temperature and light intensity (Juneja *et al.,* 2013).

The maximum carbohydrate was 1.018 µg/ml during 14th day with 10mg/L NO_3 treatment, while the minimum was 0.375 µg/ml during 14th day with 20mg/L (Fig. 13).

The maximum value of carbohydrate was 1.162 µg/ml during 14th day with 1 mg/L of PO_4 treatment, while

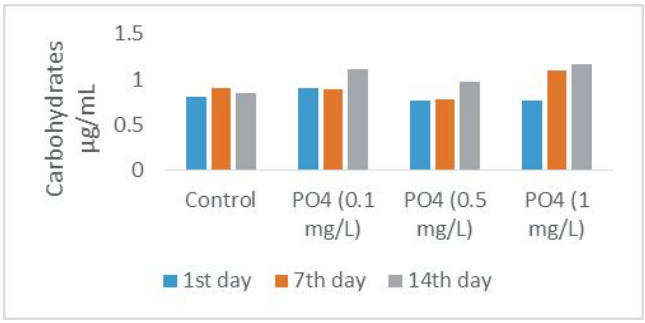

Fig. 14: Carbohydrates concentration in different concentration of PO_4 treatment.

the minimum was 0.767 µg/ml during 1st day with 0.5 and 1 mg/L (Fig. 14). In our study, it has been shown that the increase in nitrates leads to a decrease in carbohydrate concentrations over time, especially with 15 mg/L and 20 mg/L, which may be due to the fact that the scarcity of nitrogen caused an accumulation of carbohydrates and the opposite is true (Paes *et al.,* 2016).

As we observed in results that there were no different in carbohydrates in control over time and there are a slit increased in 0.1 mg/L treatment and significant increasing 14th day for all of treatments that because decreasing of algal activity and gaining carbohydrates (Paes *et al.,* 2016).

Conclusions

1. The study showed that an algae Chlorella vulgaris has a high efficiency to biorespond to an environmental impact assessment and it is possible to use algae in many cases, for example, as an environmental impact assessment tool or environmental health.
2. This Study showed can be use the bioresponse as a bio indicator for polluted environment to study environmental factors.
3. It is possible to use an organism and exploit the bioresponse that occurs to it and the changes that occur in algae as an evidence to study the environmental impact.
4. This study showed uses the biological response to study the environmental impact, by studying effect of nutrients on algae by measuring the growth rate of algae and doubling time, as well as the study of chlorophyll-a, carotenoid, proteins, carbohydrates.

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