Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 25(2): 487 – 508 (2021)
www.ejabf.journals.ekb.eg

Comparison between the toxicity of Copper and Irgarol 1051 as two different generations of antifoulings on growth and essential metabolites of marine algae (*Dunaliella salina* as a case study)

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ARTICLE INFO
Article History:
Received: Nov. 12, 2020
Accepted: March 30, 2021
Online: April 18, 2021

Keywords:
Copper;
Irgarol 1051;
Dunaliella salina;
protein;
carbohydrates;
antifoulings.

ABSTRACT

The present study aims to compare the toxicity effect of Copper and Irgarol 1051 as antifouling agents on metabolites products in marine algae *Dunaliella salina*. Different concentrations of Cu (5, 15 & 25 mg/l) and Irgarol 1051 (0.012, 0.025 & 0.050 µg/l) were laboratories prepared and their impacts on growth, protein, and carbohydrates in *Dunaliella salina* were tested. Selections of these concentrations were initiated based on the response of the studied alga which had slightly or marked effects on its growth, and also to avoid the non-effective and directly lethal concentrations on the experimented alga. The obtained results showed stimulation in the growth of algae under the effect of the lowest concentration of the two tested antifoulings, however, there was a suppression of algal growth by increasing the concentration of the two antifoulings but with different degrees. There was a clear inhibition of protein and carbohydrate production by increasing the concentration of the two tested antifoulings. Also, Irgarol 1051 showed a toxic effect higher than Copper, which is one of the essential micronutrients in the marine ecosystem.

INTRODUCTION

The term biofouling is commonly employed to distinguish the undesirable accumulation of microorganisms such as bacteria, fungi and microalgae, plants and invertebrates on any artificial surfaces submerged in seawater (**Yebra *et al*., 2004**) from those occurring on rocks and other natural objects. The organism occurring in fouling have been recorded frequently, nearly 2000 species of animals and plants have been reported from fouling. This included 615 kinds of plants and 1361 varieties of animals. Although the number of species reported from fouling is large and is widely distributed among the existing groups of organisms, it actually includes a very small proportion of the known marine species. However, adsorption and accumulation of marine organic (e.g. algae, worms and

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mollusks) to marine equipment (e.g. vessel, pontoons, piers and pipelines) can aggravate wear and lead to lower running speed, higher energy consumption and poorer operability, which may pose a great danger of sailing (**Almeida, 2007).** In addition, these marine infrastructures that are submerged in seawater are much easier suffered from corrosion. Corrosion and fouling become the major problems to work out. Besides, anti-fouling materials and anti-corrosion materials have been widely researched and used (**Xuebing *et al.*, 2018)**.

In antifouling marine paints typically up to 40% (wt.), Cu (largely in the form of Cu (I) oxide) is being used. Excessive Cu concentrations observed along the coastal and harbor areas of different parts of the world are mainly due to Cu based AF paints **(Valkirs *et al.*, 2003)**. Especially, in enclosed marinas and harbors where tidal influence is less, the Cu levels are expected to be very high. This may lead to elimination of susceptible microalgal species causing phenomenal changes like incidence of monospecific fouling communities (**Chi *et al*., 2006)**.

Today, most antifouling paints are based on copper compounds, e.g. cuprous oxide **(Amara *et al.*, 2018).** Copper is an essential element for all living organisms and play important roles in many metabolic processes **(Ochoa-Herrera *et al.*, 2011).** However, copper may also be toxic to most species when concentrations exceed levels that are physiologically required **(Strivens *et al*., 2020)**.

Copper is found naturally in the marine environment and is an essential element in the enzyme activity necessary for healthy metabolic functioning as well as the growth and metamorphosis of many organisms (**Lewis and Cave, 1982**). Copper becomes toxic when, in a bioavailable form, it exceeds the threshold of the organism's tolerance, and this has been shown to vary widely between life stages (**Xie *et al.*, 2005**) and even between species within the same taxonomical group (**Han *et al*., 2008**). Copper toxicity is also strongly influenced by environmental factors that govern copper speciation and hence its bioavailability (**Srinivasan and Swain, 2007**).

Natural background concentrations of copper in seawater are estimated between 0.5 and 3 µg/l, but concentrations up to 21 µg/l Cu have been found in contaminated areas. A recent risk assessment on the use of copper as a biocide in antifouling paints considered the concentration, speciation and effects of copper in the coastal marine environment, and inputs from AF paints. Copper toxicity was a potential problem in isolated water bodies, such as enclosed marinas and harbours with little water exchange and high levels of boating activity and recommended ongoing development of improved and environmentally friendly AF products that would reduce copper usage (**Brooks and Waldock, 2009**).

Irgarol 1051 (2-methylthio -4-terbulylamino -6- cyclopropylamino -s-triazine) is used in aquatic antifouling paints after the restriction of using tributyltin (TBT) as antifouling agent (**Zamora-Ley *et al*., 2006**). Irgarol may affect non-target photosynthetic organisms

such as phytoplankton, periphyton and aquatic macrophytes when leaching into the marine environment **(Dahl and Blanck, 1996; Hall *et al.*, 1999)**.

The active ingredients in the paints biocides (mainly copper and organostanous compounds) leach from the coating of ships and thereby contaminate the aquatic environment (**Konstantinou and Albanis, 2004**). Thus, in areas with high boating activities many of these leached substances (biocides) occur as mixtures. Occurrence of Irgarol 1051 has been widely reported in coastal waters of many countries; Red Sea, Mediterranean Sea, Japan, Australia, Europe, United States and many others (**Biselli *et al.*, 2000; Key *et al.*, 2008**).

Increasing concentration of Irgarol in coated waters up to 0.048 to 0.068 µg/l have been reported by **Bascher *et al.* (2002)**. Concentrations of Irgarol 1051 have been found to be highest and adjacent to marines and fishing harbours range between 0.02 and 0.70 µg/l, the toxic effects of Irgarol 1051 against algae, crustaceous and fish have been reported by **Van Wezel and Van Vlaardingen (2004)**.

Readmann *et al.* (2004) reported toxic effects of Irgarol 1051 at low concentrations of 0.07 µg/l. Irgarol 1051 was firstly reported as an aquatic contaminant in the Mediterranean since 1993 (**Readmann *et al.*, 1993**). Knowledge about the fate of these toxic chemicals in aquatic environments (fresh or marine) is essential for understanding and predicting the possibility of ecotoxic ecology effects.

In this research, *Dunaliella salina* was selected for the study due to different important reasons. Species of the genus *Dunaliella* have been the subject of extensive research on their physiology, biochemistry, ecology, molecular biology, and for commercial applications. *Dunaliella* species occur in hypersaline to brackish water habitats from the tropics to Antarctica. The microalga *Dunaliella salina* is the best commercial source of natural β- carotene and can accumulate significant amounts of valuable fine chemicals such as carotenoids, glycerol, lipids, vitamins, minerals, carbohydrates and proteins. Also, different species of *Dunaliella* have a large potential for biotechnological processes such as expressing of foreign proteins and treatment of wastewater (**Hosseini, 2009**). Microalgae such as *Dunaliella salina*, *Haematococcus pluvialis* and *Spirulina* sp. are also used as a source of natural pigments for the culture of prawns, salmonid fish and ornamental fish. *Dunaliella* sp., *Chlorella* sp. and *Spirulina* sp. are three major types that have been used successfully to produced high concentrations of valuable compounds such as lipids, protein, carbohydrates and pigments. Present study aims to compare toxicity effect of Copper and Irgarol 1051 as antifouling on growth and the production of essential metabolites (protein and carbohydrates) in marine algae *Dunaliella salina*.

MATERIALS AND METHODS

A- Biological material

The selective biological material is the unicellular microalga *Dunaliella salina* usually used for fish feeding; obtained from the culture collection of Botany department, Faculty

of Science, Alexandria University. The axenic cultures of *Dunaliella salina* was grown on MH medium (**Loeblich, 1982)**. The cells of 8-10 days old cultures were harvested by centrifugation at 10.000 r.p.m. for 20 min using angle rotor centrifuge. The supernatants were discarded and the remaining pellets were used for the determination of growth, protein and carbohydrates. The growth of the investigated alga was determined by cell count using the hemacytometer slide. The growth was calculated by using the formula proposed by **Stein (1973)**.

B- Heavy metal bioassay:

Stock solutions of the selected heavy metal were prepared from its salt in double distilled water and sterilized by filtration through 0.2 µm nitrocellulose membranes. According to **Wong and Pak (1992),** a preliminary experiment using a wide range of metal solutions [$CuSO_4.5H_2O$] was carried out to determine the suitable concentrations of the metal salts which could be tolerated by the studied alga. Selections of these concentrations were based on the response of the studied alga which had a slightly or marked effects on its growth, and also to avoid the non-effective and directly lethal concentrations on the experimented alga.

C- Preparation of Irgarol:

The herbicide triazine (Irgarol 1051) was purchased from Fluka Company. The stock solution 1000 mg of the standard Irgarol 1051 was prepared in acetone and stored in dark at 4°C. 0.1 ml of stock solution was mixed with 100 ml of sterilized distilled water. The mixtures were stirred by a magnetic stirrer for half an hour. Dilution of this stock solution was done by the selected culture medium for preparing the different concentrations of Irgarol. These concentrations were selected after several experiments to select the suitable concentrations. These concentrations were nearly within the range of those found in marines.

D- Determination of total protein and its fractions:

In this investigation protein was determined by the method described by **Hartree (1972)** which is the modification of the original folin-phenol method of **Lowery *et al*., (1951).** The method depends on reaction of protein with copper in alkali, then reduction of the phosphomolybdic reagent by the copper treated protein. The protein content was calculated from a standard curve made from working standards of similarly treated bovin serum albumen as mg/L culture.

***Preparation of algal extract for protein analysis:**

Protein was extracted from the algal cells according to the method described by **Rauch, (1981)** in a test tube 3-ml of 0.5 N NaOH were added to the cell pellets obtained by centrifugation from 5-ml culture, shaked well and extracted at 80˚C in a water bath for 10 minutes with occasionally stirring. The extract was quickly cooled at room temperature (using running cold water), centrifuged at 5000 rpm for 20 minutes and the supernatant was transferred to a graduated cylinder (10 ml).

Extraction was repeated two times with 0.5 N NaOH for 10 min at 80˚C. The combined supernatants were completed to a definite volume (10 ml) mixed well and used for protein measurement.

***Total Protein estimation:**

Reagents:

- **Reagent A:** two gm potassium sodium tartarate and 100g sodium carbonate dissolved in 1 liter of 0.5 N NaOH (0.9 ml used for each determination)**.**
- **Reagent B:** two gm potassium sodium tartarate and 1.0 g $CuSO_4.5H_2O$ dissolved in 100 ml 0.1 N NaOH (0.1 ml was used each determination)**.**
- **Reagent C:** one volume Folin ciocalteu reagent diluted ten fold with distilled water (3.0 ml were used for each determination)

Procedure:

Reagent A was added first to one ml centrifuged clear extract and to the blank or standard solution in a test tube and heated in water bath at 50˚C for 10 min. cooled rapidly in ice. Reagent B was then added and the tube was left for at least 10 min at room temperature. Reagent C was then added and mixed well by whiler mixer, placed in water bath at 50˚C for 10 min., then cooled rapidly. The intensity of the blue colour developed in each sample was measured using a spectrophotometer (Berkin –Elmer) at 650 nm and readings were then related to the standard curve.

***Determination of water soluble proteins:**

Aliquots (10 mls) of algal suspension were centrifuged. The algal precipitate was boiled in 10 mls distilled water for two hours. After cooling, the water extract was centrifuged and the supernatant was decanted and completed to a definite volume by distilled water and analyzed for determination of soluble proteins according to the method of total protein content.

*** Determination of water-insoluble proteins:**

It was calculated as the difference between the total and water-soluble protein fractions of the same sample.

E- Measurements of carbohydrates content:

Carbohydrates content were estimated according to the method described by **Dubois *et al*., (1959).**

***Determination of total carbohydrates:**

In a test tube two mls of N HCl were added to the-cell pellets -obtained from 5ml culture after centrifugation and shaken well for complete homogeneity. The mixture was transferred to a clean test tube and then boiled for 20 min. (a time was found to be enough for complete hydrolysis) in a boiling water bath for complete hydrolysis. After cooling at room temperature, the extract is clarified by centrifugation for five minutes at 2000 rpm. In a clean test tube 0.5 ml of the clear supernatant and 0.5 ml of 5% phenol solution was added and mixed well. To each tube 2.5 ml of 98% H_2S0_4 were added rapidly so that the stream hit the liquid surface directly to produce good mixing. Tubes were equally

agitated during the acid addition. Blank was also treated in the same manner, but 0.5 ml distilled water was used instead of algal extract. After ten minutes, the tube were reshaken and placed in a water bath at 25-30°C for 20 min. The developed yellow-orange color was stable for several hours. The optical density was determined at 488 nm and compared to calibration standard curve.

***Determination of water- soluble carbohydrates:**

To estimate water-soluble carbohydrates, 5 ml culture from each algal culture was centrifuged and the supernatant was decanted. After decantation, the algal precipitate was extracted by distilled water for two hours in a boiling water bath. After cooling, the soluble fraction was separated by centrifugation and completed to a definite volume. Then the water soluble carbohydrates were determined by the method mentioned previously in case of total carbohydrates.

***Determination of water-insoluble carbohydrates content:**

It was calculated as the difference between the total carbohydrates and the water soluble carbohydrates of the same sample.

F- Statistical analysis:

The obtained data were analyzed statistically using two ways ANOVA (Analysis of variance). The difference between means probability levels were analyzed using Duncan's New Multiple Range Test ($P< 0.05$). F- test were also analyzed (LSD) the least significant difference at 0.05.

RESULTS AND DISCUSSION

1-Growth

Cu is having an essential role in the algal metabolism **(Thompson *et al.*, 1987; Lage *et al.*, 1996).** However, in higher concentrations Cu tends to damage the cell wall membrane function, **Chi *et al.*, (2006)**. Copper is also universally used as biocide as it is lethal to microorganism at higher concentrations **(Rhie and Lee, 1999)**. Copper concentrations less than 1 μg/l retard the growth of fresh and marine microalgal species **(Davey *et al.*, 1973)**. Coatings containing increasing cuprous oxide concentrations that were exposed in a pleasure craft marina in Gothenburg, i.e. in the region Baltic Transition **(Lindgren *et al.*, 2018)**. All investigated copper coatings were efficient in preventing macrofouling and copper was determined to be ≤ 4.7 μg/cm2/d, indicating that coatings with even lower release rates of copper may be sufficient in preventing macrofouling.

It is clear from the results recorded in Table (1) and Figures (1-A&B) that maximum growth value of *Dunaliella salina* was reached at the 8^{th} day of culturing. This is coincident with the results obtained by **Ben-Amotz and Avon (1981), El-Maghrabi, (1997)** and **Sahar, (1998)**. Low concentration of Cu^{2+} ions (5mg/l) stimulates the growth of this organism compared to control. Further with the increase in concentration of Cu^{2+} ions (15 , 25 mg/l), the cell number decreased gradually till the end of the experiment

compared to concentration 5 mg/l. Event at the last two days of culturing (12-14 day) the organism disappeared completely at concentration 25mg/l.

Anent the effect of different concentration of Irgarol 1051 viz 0.012, 0.025 and 0.050 μg /l on the growth of *Dunaliella salina* cultured for 14 days revealed that, gradual increase in number of cells till the end of the experiment in case of concentration 0.012 μg/l, percontra at concentrations 0.025 and 0.050 μg/l was great decrease in number of cells after 6^{th} day till the end of the experiment compared to concentration 0.012 μg/l. There was suppression of algal growth under the effect of the different tested concentrations of Irgarol may be due to the increasing of toxicity of this biocide. The same results were also obtained by **Munnas (2003); Singla and Garg (2005).** Our results would contradict the study of **Gatidou *et al.* (2003)** who suggested that Irgarol had a lethal effect on *Dunaliella tertiolecta* and it inhibits growth at concentration higher than 0.8 µg/l and at concentration 3.0µg/l, the compound killed almost all the cells, also he found that, *Dunaliella tertiolecta* showed a 50% decrease in growth rate during a 96 h exposure to about 3.95 nM of Irgarol while at 11.84 nM almost all cells were killed (96% inhibition). The lethal effect of Irgarol was furthermore described in the **ACE (2002)** final report in which flow cytometric analyses revealed that approximately half of the phytoplankton was killed at concentrations as low as 0.39 nM (100 ng/l^{-1}) **(Anita *et al.*, 2009)**.

Tsang *et al*. (1999) revealed that, sensitivity and response of microalgae to booster biocides varies from species to species, size of the cell wall composition, consequently some species appeared to be resistant to booster biocides and posses the ability to accumulate and/or degrade these compounds.

2- PROTEIN

A glance of result recorded in Table (2-A) and graphed in Figure (2-A), concerning the effect of different concentrations of Cu^{2+} and Irgarol 1051 on the protein fraction content (soluble, insoluble and total) in *Dunaliella salina* cultured for 16 days. Concentration of 5 mg/l Cu^{2+} caused stimulatory effect of increasing protein content till the 8^{th} day of culturing then decreasing in the protein content soluble, insoluble and total) noticed till the end of the experiment. On the contrary at concentrations 15 and 25 mg/l of Cu^{2+} a noticeable decrease in the content of soluble, insoluble and total protein could be observed from the first 4 days till the end of the experiment. At concentration 25 mg/l of Cu^{2+}, there was a sharp decrease in protein content (soluble, insoluble and total) higher than in case of concentration 15 mg/l of Cu^{2+} , and after the 12^{th} day there was no protein detected, that's mean concentration 25 mg/l of Cu^{2+} was highly toxic to this type of alga. Similar observations were also reported by **El-Agawany, (2008)** in case of *Dunaliella tertiolecta*. The importance of Cu^{2+} as an essential micro-nutrient and its effect in limiting algal growth was reported by many authors (**Steeman-Nielsen and Wium-Anderson 1970**; **Wong and Chang, 1991** and **Abdel-Hamid *et al*., 1992; Vymazal, 1995**). Also, **Stauber and Florence (1985a)** reported that copper at concentrations higher than 5 mg/l

inhibited the growth of *N. closterium* by 50% below control. Moreover, the toxic effect of copper on the growth of the marine alga *Dunaliella tertiolecta* was clearly demonstrated in the cultures treated with 10 and 12 mg/l copper as recorded by **Abalade *et al*., (1995b)**.

Table (1): Number of cells X10^6 of *Dunaliella salina* cultured for 14 days at different concentrations of Cu^{2+} ions (mg/l) and at different concentrations of Irgarol 1051 (μg/l).

Time (Days)	Conrrol	Cu concentrations (mg/l)			Irgarol concentrations (μg/l)			F (p)	LSD
		5	15	25	0.012	0.025	0.050		
0	0.46±0.002^a	0.46±0.002^a	0.46±0.002^a	0.46±0.002^a	0.46±0.002^a	0.46±0.002^a	0.46±0.002^a	0.000 (1.000)	0.003
2	0.53±0.002^a	0.53±0.003^a	0.53±0.003^a	0.52±0.003^c	0.53±0.001^a	0.53±0.003^a	0.46±0.004^c	37.059* (<0.001)	0.003
4	0.74±0.007^a	0.78±0.001^b	0.66±0.003^c	0.64±0.001^a	0.67±0.02^a	0.53±0.01^b	0.51±0.013^c	812.857** (<0.001)	0.004
6	1.23±0.03^a	1.30±0.003^b	1.18±0.002^c	0.91±0.003^f	1.00±0.004^b	0.64±0.004^b	0.57±0.005^c	15207.000** (<0.001)	0.003
8	2.23±0.02^a	2.84±0.002^b	1.54±0.004^a	0.75±0.004^e	2.05±0.006^b	0.70±0.008^b	0.87±0.008^c	31197.000** (<0.001)	0.005
10	2.93±0.002^a	3.14±0.001^b	2.55±0.002^c	0.63±0.054^e	2.11±0.004^b	0.70±0.04^b	0.67±0.013^d	57015.000** (<0.001)	0.004
12	3.33±0.02^a	3.35±0.003^b	2.74±0.001^d	0.35±0.001^f	2.45±0.007^b	0.79±0.015^c	0.71±0.019^c	225033.191** (<0.001)	0.004
14	2.58±0.04^a	2.49±0.003^b	0.92±0.004^d	---	1.63±0.01^b	0.75±0.005^c	0.07±0.011^d	370898.571** (<0.001)	0.004

F (p): F-test (ANOVA) and its significance between groups. LSD: Least significant difference at 0.05.
* : Statistically significant at $p \leq 0.05$. **: Statistically significant at $p \leq 0.01$.
Different subscribts are significant . Data are expressed in mean ±SD

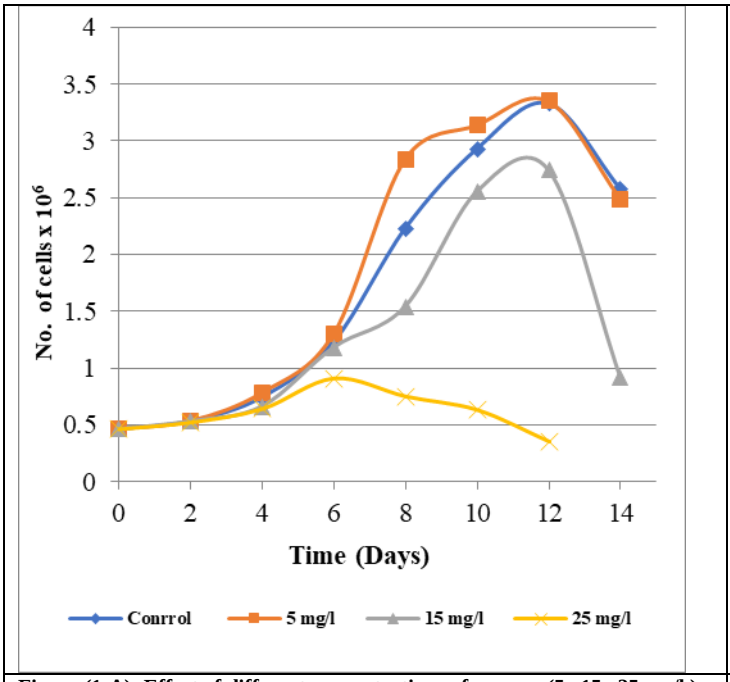


Figure (1-A): Effect of different concentrations of copper (5 , 15 , 25 mg/l) on growth of *Dunaliella salina* cultured for 14 days.

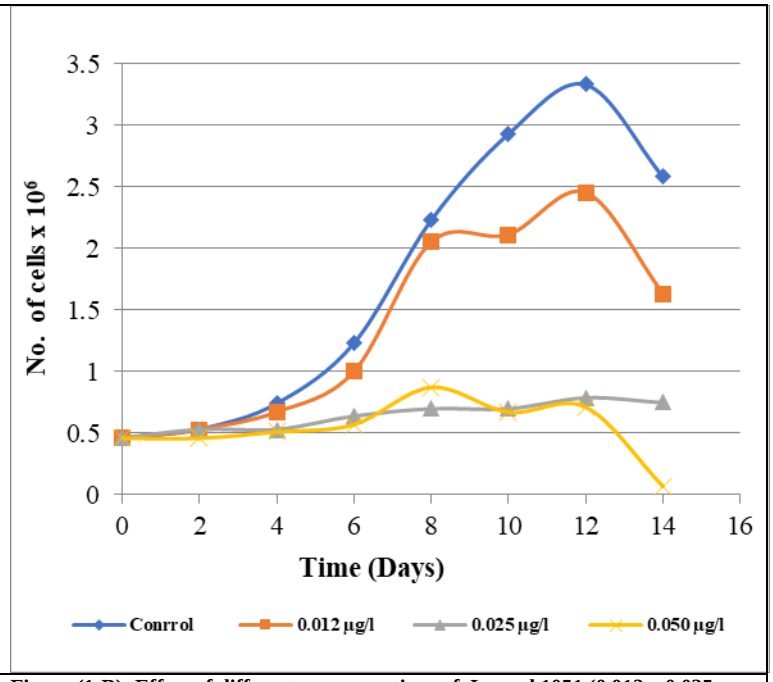


Figure (1-B): Effect of different concentrations of Irgarol 1051 (0.012 , 0.025 and 0.050 µg/l) on growth of *Dunaliella salina* cultured for 14 days.

Owing to these data recorded in Table (2-B) and graphed in Figure (2-B) concerning the effect of different concentration of the antifouling Irgarol 1051 on the content of protein fractions of *Dunaliella salina,* it is clear that, the content of total proteins at the untreated cultures (control), and at concentration 0.012 µg/l Irgarol 1051 increased gradually till the end of the experiment. However, the rate of increase in case of concentration 0.012 µg/l Irgarol 1051 was less than in case of control. In case of the other two concentrations (0.025& 0.050 µg/l), total protein content reached its maximum value at the 8^{th} day of culturing but it was higher in case of concentration 0.025 µg/l than concentration 0.050 µg/l (34.501 & 26.470 mg/ml respectively).

The decrease in the content of total proteins at the end of the experiment may be due to decrease in insoluble concentration and deficiency of nutrients which increase protolysis **(Cooke *et al.*, 1979 and 1980)** and/or to decrease in the rate of protein synthesis **(Vaodia and Waisal, 1967)**. Although total proteins content decreased under the stress effect of Irgarol yet protein groups differed under the stress effect of the different levels of Irgarol. Soluble proteins increased by increasing the concentration of Irgarol, while insoluble one decreased, i.e. the greater the toxic effect of the antifouling agent Irgarol and the length of culturing period, the greater the content of soluble proteins and the less of the content of insoluble fraction, This means that, the increase in the soluble proteins and the decrease in the insoluble one may be due to the degradation in insoluble fractions to soluble one or may be due to the decrease in the synthesis of insoluble one and to the accumulation of the soluble proteins under the toxic stress of this antifouling agent. It must be mentioned that, the ability of Irgarol degradation was considered to be species dependent upon expense to Irgarol. The first phase would be its rapid biosorprtion onto the cell surface. This antifouling compounds then accessed to the cell interior through diffusion or via some ion channels as proposed by **St-louis *et al*., (1997)**.

Table (2-A): Content of protein fractions (mg/ml) in *Dunaliella salina* cultured for 16 days under the effect of different concentrations of Cu^{2+} (5 , 15 , 25 mg/l).

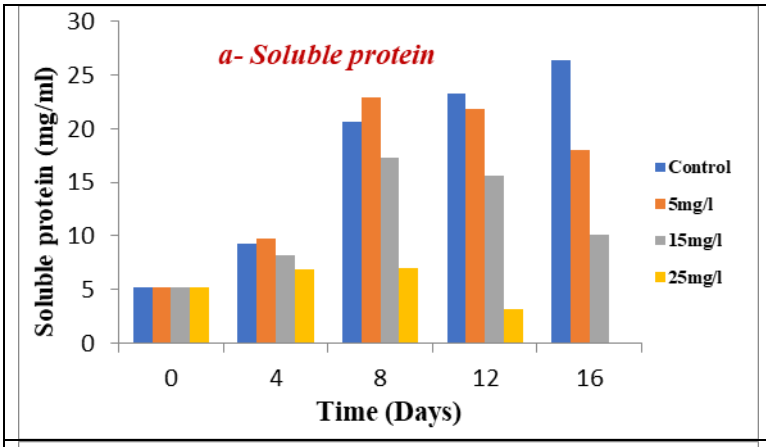
Time (Days)	Parameter	Control	Different concentrations of Cu^{2+} (mg/l)			F (p)	LSD
			5	15	25		
0	Soluble	5.249±0.002 [a]	5.249±0.002 [a]	5.249±0.002 [a]	5.249±0.002 [a]	42024.884** (<0.001)	0.002
	Insoluble	6.204±0.002 [a]	6.204±0.002 [a]	6.204±0.002 [a]	6.204±0.002 [a]	290036.34** (<0.001)	0.002
	Total	*11.453*	*11.453*	*11.453*	*11.453*		
4	Soluble	9.221±0.002 [a]	9.734±0.001 [b]	8.196±0.002 [c]	6.849±0.004 [e]	189355.03** (<0.001)	0.003
	Insoluble	10.226±0.002 [a]	10.793±0.002 [b]	8.842±0.002 [c]	7.850±0.001 [f]	42666.09** (<0.001)	0.002
	Total	*19.447*	*20.527*	*17.038*	*14.699*		
8	Soluble	20.625±0.004 [a]	22.963±0.003 [b]	17.325±0.004 [d]	6.941±0.004 [f]	14105523** (<0.001)	0.003
	Insoluble	22.161±0.001 [a]	25.045±0.004 [b]	19.187±0.001 [d]	7.458±0.001 [f]	21381462** (<0.001)	0.003
	Total	*42.786*	*48.008*	*36.512*	*14.399*		
12	Soluble	23.344±0.003 [a]	21.852±0.001 [b]	15.582±0.002 [d]	3.198±0.002 [f]	34034526** (<0.001)	0.003
	Insoluble	26.246±0.005 [a]	26.297±0.003 [b]	20.788±0.005 [d]	3.595±0.001 [f]	47651325** (<0.001)	0.003
	Total	*49.608*	*48.149*	*36.370*	*6.793*		
16	Soluble	26.410±0.004 [a]	18.061±0.001 [b]	10.102±0.001 [d]	---	$1032x10^{8}$** (<0.001)	0.002
	Insoluble	30.319±0.007 [a]	24.660±0.004 [b]	13.952±0.002 [d]	---	60366139** (<0.001)	0.004
	Total	*56.729*	*42.721*	*24.054*	---		

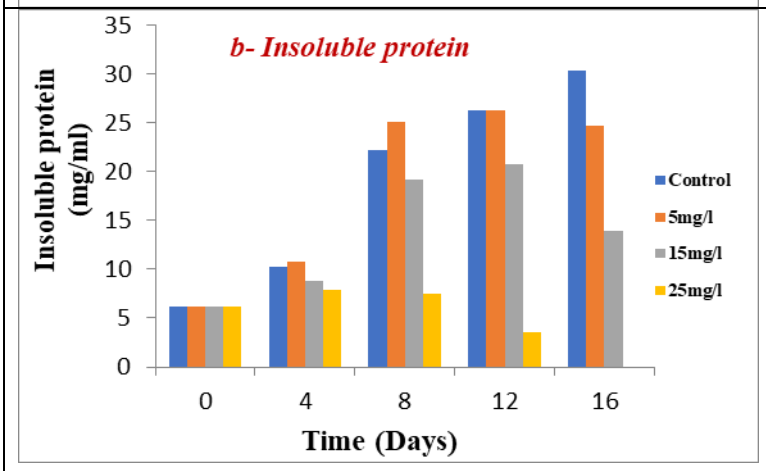
F (p): F-test (ANOVA) and its significance between groups.
LSD: Least significant difference at 0.05.
*** : Statistically significant at p ≤ 0.05.**
****: Statistically significant at p ≤ 0.01.**
Different subscribts are significant .
Data are expressed in mean ±SD

Table (2-B): Content of protein fractions (mg/ml) in *Dunaliella salina* cultured for 16 days under the effect of different concentrations of Irgarol 1051 (0.012 , 0.025 and 0.050 µg/l).

Time (Days)	Parameter	Control	Irgarol 1051 concentrations (µg/l)			F (p)	LSD
			0.012	0.025	0.050		
0	Soluble	5.249±0.002 a	5.249±0.002 a	5.249±0.002 a	5.249±0.002 a	42024.884** (<0.001)	0.002
	Insoluble	6.204±0.002 a	6.204±0.002 a	6.204±0.002 a	6.204±0.002 a	290036.34** (<0.001)	0.002
	Total	*11.453*	*11.453*	*11.453*	*11.453*		
4	Soluble	9.221±0.002 a	11.070±0.001 b	10.583±0.001 c	11.029±0.003 d	19895819** (<0.001)	0.003
	Insoluble	10.226±0.002 a	7.055±0.003 b	8.274±0.003 c	5.561±0.014 d	29393334** (<0.001)	0.002
	Total	*19.447*	*18.125*	*18.857*	*16.590*		
8	Soluble	20.625±0.004 a	14.081±0.004 b	20.051±0.002 c	16.420±0.007 d	31577920** (<0.001)	0.003
	Insoluble	22.161±0.001 a	16.201±0.001 b	14.450±0.003 c	10.050±0.004 d	40250405** (<0.001)	0.003
	Total	*42.786*	*30.282*	*34.501*	*26.470*		
12	Soluble	23.344±0.003 a	25.260±0.002 b	26.030±0.005 c	16.551±0.021 d	61389579** (<0.001)	0.002
	Insoluble	26.246±0.005 a	15.222±0.004 b	12.468±0.021 c	7.515±0.070 d	40303137** (<0.001)	0.004
	Total	*49.608*	*40.482*	*38.498*	*24.066*		
16	Soluble	26.410±0.004 a	24.200±0.001 b	20.520±0.004 c	15.210±0.041 d	14105523** (<0.001)	0.003
	Insoluble	30.319±0.007 a	21.556±0.003 b	16.604±0.007 c	11.233±0.052 d	21381462** (<0.001)	0.003
	Total	*56.729*	*45.756*	*37.124*	*26.443*		

F (p): F-test (ANOVA) and its significance between groups.
LSD: Least significant difference
at 0.05. * : Statistically significant at p ≤ 0.05.
****: Statistically significant at p ≤ 0.01. Different subscribts are significant.**
Data are expressed in mean ±SD





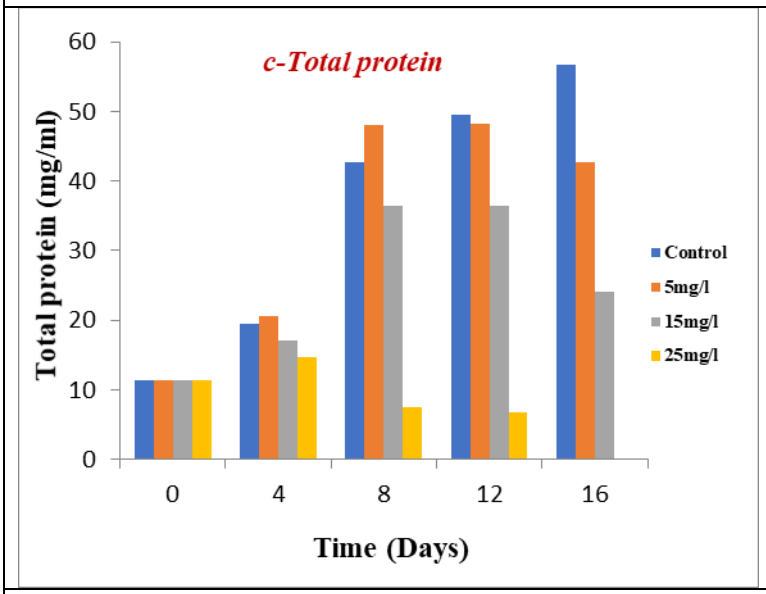
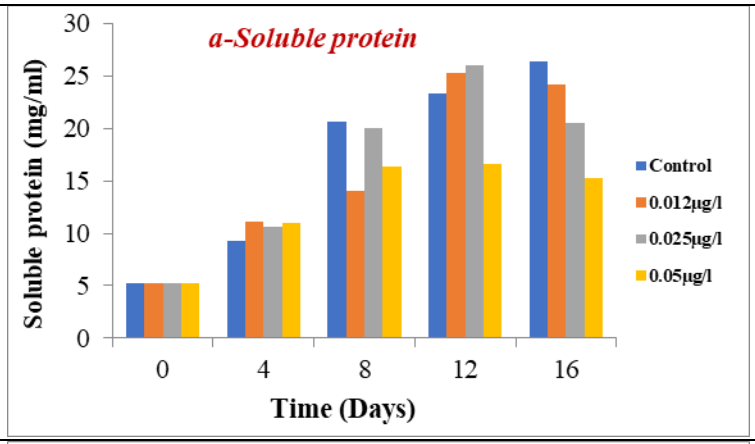
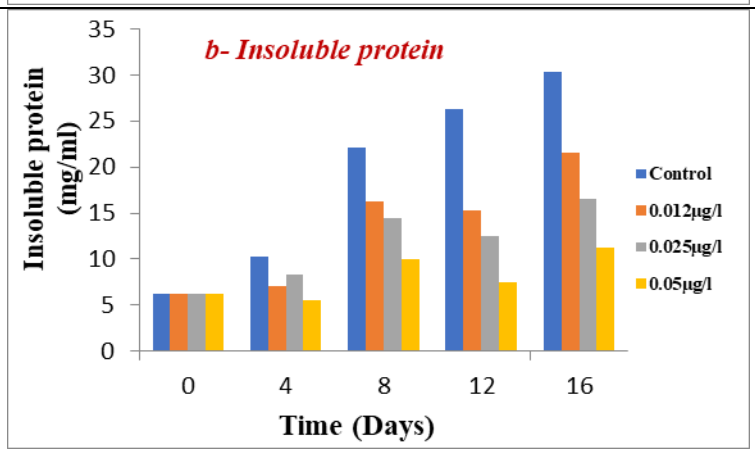


Figure (2-A): Content of protein fractions (mg/ml) in *Dunaliella salina* cultured for 16 days under the effect of different concentrations of Cu^{2+} (5 , 15 , 25 mg/l).





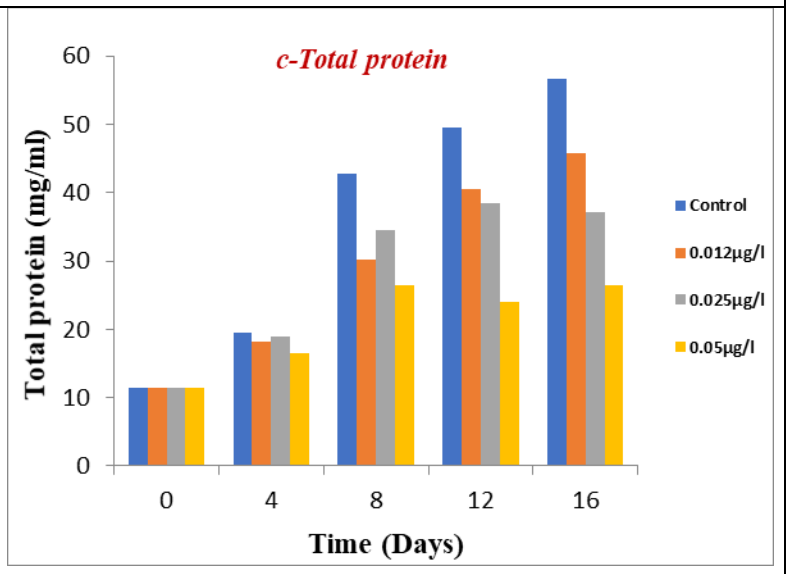


Figure (2-B): Content of protein fractions (mg/ml) in *Dunaliella salina* cultured for 16 days under the effect of different concentrations of Irgarol 1051 (0.012 , 0.025 and 0.050 µg/l).

3-Carbohydrates

A glance of result recorded in Table (3-A) and graphed in Figure (3-A), concerning the effect of different concentrations of Cu^{2+} and Irgarol 1051 on the carbohydrate content (soluble, insoluble and total) in *Dunaliella salina* cultured for 16 days, it is clear that , in case of 5mg/l of Cu^{2+} the total content of carbohydrates recorded the same value compared to control at the first 4 days of culturing, then the contents of total carbohydrates increased over control till the 12^{th} day of culturing. However, at the 16^{th} day of culturing the content of carbohydrates increased more than at the 12^{th} day of culturing, yet the value obtained was less than control. On the contrary at concentrations 15 and 25 mg/l of Cu^{2+}, the toxic effect of Cu^{2+} ions increases gradually with increasing the concentration of element and period of culturing. At the 16^{th} day of culturing , total carbohydrate content was undetected at concentration 25 mg/l due to highly toxic effect .

As previously reported, at low concentrations of Cu^{2+}, the stimulation of growth of *Dunaliella salina* was accompanied by an increase in total soluble carbohydrates. It is worth mentioning here that the movement of copper into cell is believed to occur mainly by non metabolic transport and the plasmalemma is the initial site of copper binding in the sequence leading to intracellular copper uptake thus, factor limiting the binding of copper to the plasmalemma or preventing further passage of copper into cells will similarly limit copper toxicity (**Vymazal, 1995**). In other studies, **Twiss and Nalewajko, (1992)** suggested that polyphosphate plays a passive role in protecting cells from copper.

Our results go with harmony with those obtained by **Chi *et al.*, (2006)**, they found that heavy metals released from AF coatings tend to cause change in the growth and biochemical metabolism of the marine organisms.

Results obtained from the experiments recorded in Table (3-B) and graphed in Figure (3-B) that have been carried out on the effect of Irgarol on the carbohydrates content revealed that, all the tested concentrations of Irgarol were inhibitor to carbohydrates content of *Dunaliella salina*. These results go in harmony with those obtained by **Sidharthan*et al.*, (2002)** who reported that, high concentration of TBT on 1.0 ng/l, the proteins and carbohydrates were inhibited in *Nannochloropsis oculata* and bring about drastic change in its biochemical compositions.

Khodse and Meana,(2007) found that, antifouling agents like TBT and Irgarol influenced cellular and extracellular carbohydrate production. The results obtained in this investigation are in harmony with those recorded for such authors. Also, **Mishra *et al.*, (2008)** reported that, among different solute accumulating in response of stress, sugar play a key role to maintain the osmotic regulation in cells.

Table (3-A): Content of carbohydrate fractions (soluble , insoluble and total in mg/l) in *Dunaliella salina* under the effect of different concentrations of Cu^{2+} (5 , 15 and 25 mg/l) .

Time (Days)	Parameter	Control	Different concentrations of Cu^{2+} (mg/l)			F (p)	LSD
			5	15	25		
0	Soluble	5.59±0.002 [a]	5.59±0.002 [a]	5.59±0.002 [a]	5.59±0.002 [a]	386923.80** (<0.001)	0.002
	Insoluble	13.67±0.001 [a]	13.67±0.001 [a]	13.67±0.001 [a]	13.67±0.001 [a]	11450350** (<0.001)	0.002
	Total	*19.26*	*19.26*	*19.26*	*19.26*		
4	Soluble	9.69±0.002 [a]	9.69±0.001 [a]	7.23±0.004 [c]	5.57±0.003 [e]	434608.82** (<0.001)	0.003
	Insoluble	19.15±0.001 [a]	19.15±0.002 [b]	15.70±0.002 [d]	10.39±0.002 [f]	7210688.6** (<0.001)	0.002
	Total	*28.84*	*28.84*	*22.93*	*15.96*		
8	Soluble	20.01±0.003 [a]	27.14±0.003 [b]	16.99±0.002 [d]	6.73±0.001 [f]	206431.13** (<0.001)	0.023
	Insoluble	36.60±0.002 [a]	49.62±0.002 [b]	22.19±0.003 [d]	12.32±0.003 [f]	1.48×10^{8} ** (<0.001)	0.002
	Total	*56.61*	*76.76*	*39.18*	*19.05*		
12	Soluble	27.43±0.001 [a]	27.55±0.003 [b]	13.69±0.002 [d]	3.76±0.002 [f]	48892236** (<0.001)	0.003
	Insoluble	38.30±0.004 [a]	39.25±0.002 [b]	25.45±0.004 [d]	5.25±0.003 [f]	1.16×10^{8} ** (<0.001)	0.004
	Total	*65.73*	*66.80*	*39.14*	*9.01*		
16	Soluble	24.19±0.002 [a]	22.82±0.003 [b]	14.14±0.003 [d]	000±0.000 [f]	99105492** (<0.001)	0.002
	Insoluble	43.46±0.003 [a]	40.89±0.002 [b]	17.71±0.004 [d]	000±0.000 [f]	2.05×10^{8}** (<0.001)	0.003
	Total	*67.56*	*63.71*	*31.85*	*000*		

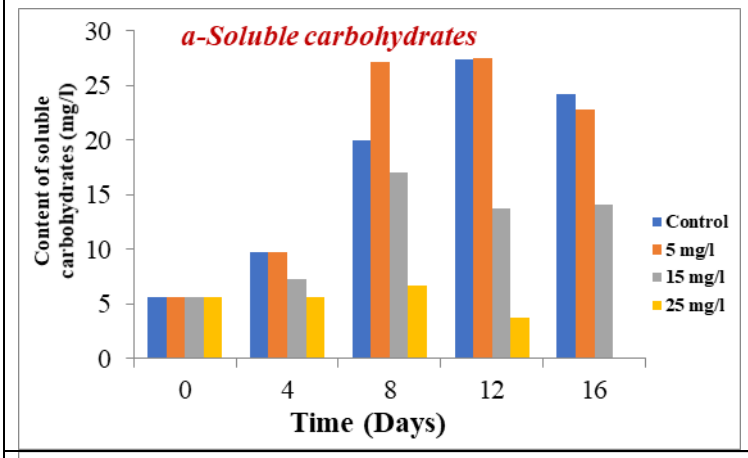
F (p): F-test (ANOVA) and its significance between groups. LSD: Least significant difference at 0.05. * : Statistically significant at p ≤ 0.05. **: Statistically significant at p ≤ 0.01. Different subscribts are significant . Data are expressed in mean ±SD

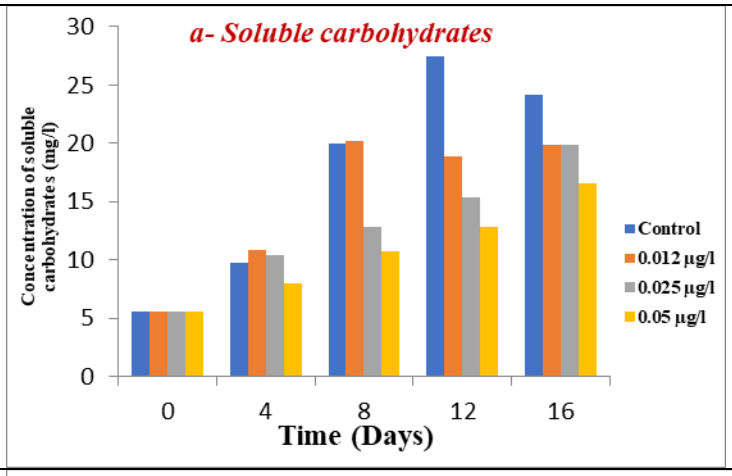
Table (3-B): Content of carbohydrate fractions (soluble , insoluble and total in mg/l) in *Dunaliella salina* under the effect of different concentrations of Irgarol 1051 (0.012 , 0.025 and 0.050µg/l) .

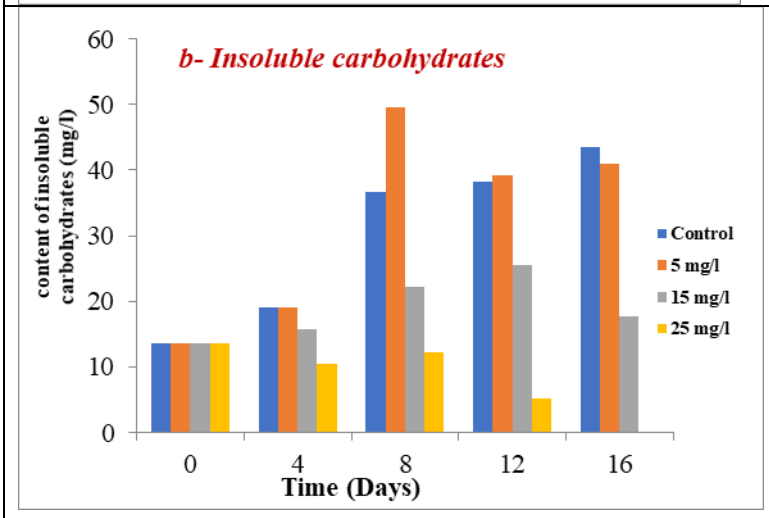
Time (Days)	Parameter	Control	Irgarol 1051 concentration (µg/l)			F (p)	LSD
			0.012	0.025	0.050		
0	Soluble	5.59±0.002 [a]	5.59±0.002 [a]	5.59±0.002 [a]	5.59±0.002 [a]	386923.80** (<0.001)	0.002
	Insoluble	13.67±0.001 [a]	13.67±0.001 [a]	13.67±0.001 [a]	13.67±0.001 [a]	11450350** (<0.001)	0.002
	Total	*19.26*	*19.26*	*19.26*	*19.26*		
4	Soluble	9.69±0.002 [a]	10.84±0.4 [b]	10.35±0.08 [c]	8.03±0.36 [d]	12766177** (<0.001)	0.003
	Insoluble	19.15±0.001 [a]	16.32±0.62 [b]	12.81±0.023 [c]	12.28±0.002 [d]	44880350** (<0.001)	0.004
	Total	*28.84*	*27.20*	*23.16*	*20.31*		
8	Soluble	20.01±0.003 [a]	20.16±0.08 [b]	12.85±0.01 [c]	10.71±0.024 [d]	88582430** (<0.001)	0.002
	Insoluble	36.60±0.002 [a]	19.54±0.05 [b]	18.92±0.005 [c]	13.26±0.003 [d]	65470628** (<0.001)	0.004
	Total	*56.61*	*39.70*	*31.77*	*23.97*		
12	Soluble	27.43±0.001 [a]	18.87±0.05 [b]	15.38±0.004 [c]	12.88±0.024 [d]	69301031** (<0.001)	0.002
	Insoluble	38.30±0.004 [a]	26.64±0.08 [b]	23.34±0.002 [c]	15.34±0.003 [d]	93391209** (<0.001)	0.004
	Total	*65.73*	*45.42*	*38.72*	*28.22*		
16	Soluble	24.19±0.002 [a]	19.85±0.09 [b]	19.90±0.002 [c]	16.55±0.11 [d]	206431.13** (<0.001)	0.023
	Insoluble	43.46±0.003 [a]	29.01±0.14 [b]	17.66±0.003 [c]	10.08±0.013 [d]	1.48×10^{8}** (<0.001)	0.002
	Total	*67.56*	*48.86*	*37.56*	*26.63*		

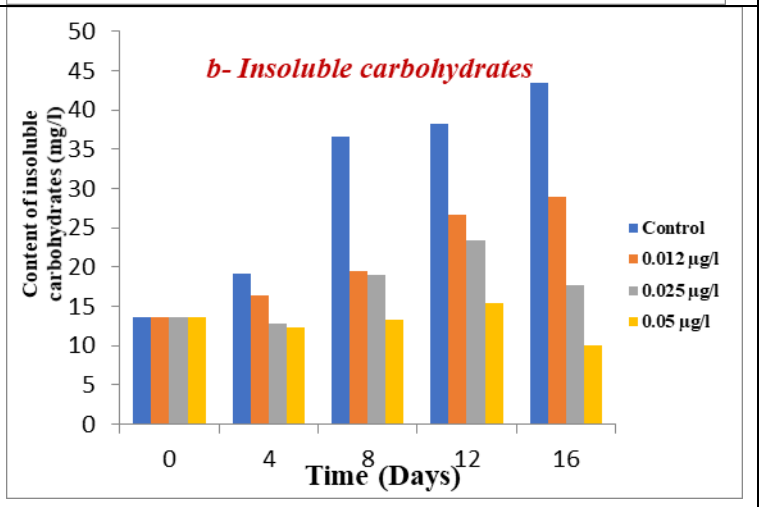
F (p): F-test (ANOVA) and its significance between groups. **LSD: Least significant difference at 0.05.**
*** : Statistically significant at p ≤ 0.05.** ****: Statistically significant at p ≤ 0.01.**
Different subscribts are significant . **Data are expressed in mean ±SD**

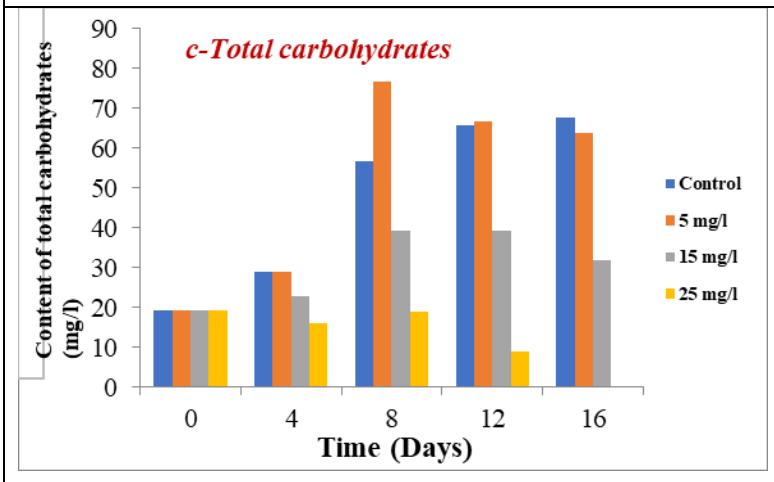
There are earlier reports on carbohydrates accumulation on response of various abiotic stress during reproductive development **(Meier and Reid, 1982)**. It was found also by **Parado *et al*. (2000)** that, accumulation of sugars is enhanced in response of verity of environmental stress. However, our work cleared that, the content of carbohydrates whether soluble, insoluble and total depended mainly on the concentration of the stress compound and the length of culturing period. This fact could be reported from the data obtained in this paper where all the carbohydrate fractions after the 16th day of culturing greatly decreased specially at the highest concentration (0.050 µg/l) and the rate of decrease depended mainly on the concentration of Irgarol. The decrease in carbohydrates content may indicate that, the efficiency of photosynthesis began to decrease owing to the destruction of chloroplast pigments **(Gale *et al.*,1966)**.











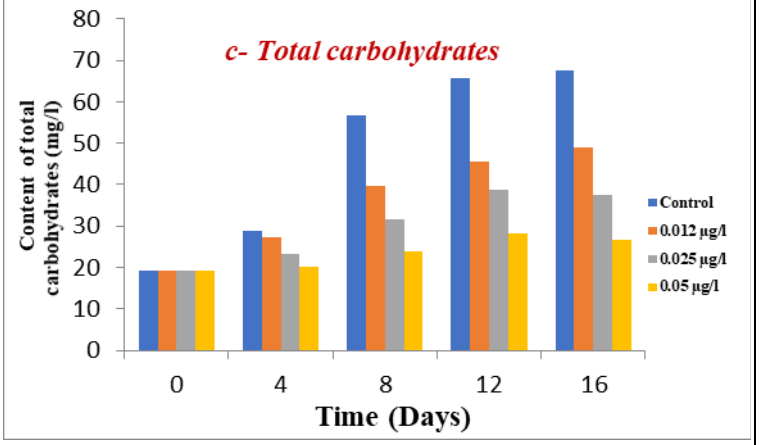


Figure (3-A): Content of carbohydrate fractions (soluble , insoluble and total in mg/l) in *Dunaliella salina* under the effect of different concentrations of Cu^{2+} (5 , 15 and 25 mg/l) .

Figure (3-B): Content of carbohydrate fractions (soluble , insoluble and total in mg/l) in *Dunaliella salina* under the effect of different concentrations of Irgarol 1051 (0.012 , 0.025 and 0.050µg/l) .

CONCLUSION

Present study aims to compare toxicity effect of Copper and Irgarol 1051 as two antifouling agents on metabolites products in marine algae *Dunaliella salina*. Different concentrations of Cu (5, 15 & 25 mg/l) and Irgarol 1051 (0.012, 0.025 & 0.050 μg/l) were laboratory prepared and their impacts on growth, protein and carbohydrates on *Dunaliella salina*. The obtained data were analyzed statistically using two ways ANOVA. The difference between means probability levels were analyzed using Duncan's New Multiple Range. Results showed that at low concentration of Cu^{2+} ions (i.e. 5 mg/l), the growth of algae stimulated compared to control. However, the cell is gradually decreased at high concentration of Cu^{2+} ions (15, 25 mg/l). In regard of Irgarol 1051, a gradual increase in number of cells was recorded till the end of the experiment with concentration 0.012 μg/l, however percontra at concentrations 0.025 and 0.050 μg/l was great decrease in number of cells after 6^{th} day till the end of the experiment compared to concentration 0.012 μg/l. There was suppression of algal growth under the effect of the different tested concentrations of Irgarol which may be due to increase toxicity of this biocide. At concentration 25 mg/l of Cu^{2+}, there was a steep decrease in protein content (soluble, insoluble and total) higher than in case of concentration 15 mg/l of Cu^{2+} and after the 12^{th} day no protein detected indicating to a toxic effect. The greater the toxic effect of the antifouling agent Irgarol and the length of culturing period, the greater the content of soluble proteins and the less content of insoluble fraction. The decrease in the content of total proteins at the end of the experiment may be due to decrease in insoluble concentration and deficiency of nutrients which induces protolysis. The toxic effect of Cu^{2+} ions increase gradually with increasing the concentration of element and period of culturing, however concentrations of Irgarol even very low were inhibitor to carbohydrates content of *Dunaliella salina.* Vulnerability of *Dunaliella salina* to Irgarol 1051 and Cu showed different responses. The damage resulted from existence of *Dunaliella* algae in marine environment with very low concentrations of Irgarol 1051 is much more than Cu. This can be explained by the high toxicity level of Irgarol, which is classified as a toxic antifouling in comparison with Cu which is one of the essential micronutrients in marine ecosystem.

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