Biological monitoring of inhibitory effects of antifouling agent Irgarol 1051 on growth and essential metabolites of marine alga Chlorella salina

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1. ABSTRACT: Irgarol 1051 (2-(tert-butylamino)-4-(cyclopropylamino)-6-(methylthio)-1,3,5-triazine) is a biocide used in antifouling paint on ships to prevent fouling growth. It is widely used booster herbicides and it may exert potent toxic effects on marine primary producers such as microalgae, where majority of the toxicity data are based on changing in growth and inhibition of essential metabolites. Data of the toxic effects on microorganisms may be used to define the upper limits for concentration of pollutants and to predict environmental toxicity risk. So, the aim of this work was concentrated on the effect of the booster biocide Irgarol 1051 on growth and content of some essential metabolites namely protein, carbohydrates, amino acids and fatty acids of marine planktonic alga Chlorella salina. The result obtained proved that, increasing of antifouling concentration caused notable decreasing in growth depending on the concentration used. The presence of antifouling agent Irgarol 1051 in the seawater especially in areas with heavy shipping activity due to its application in antifouling paints will cause decreasing in nutrient value of proteins in this type of algae as well as the negative impact on the essential amino acids. Carbohydrates content decreased by increasing the concentration of Irgarol. A serious decrease in the content of Omega-3 fatty acids and in turn this will affect the crop of the marine fish since fishes cannot synthesis these types of fatty acids.

Keywords: *Chlorella salina, Irgarol 1051, protein, carbohydrates, amino acids, fatty acids*

2. INTRODUCTION

The main cause of pollution is the discharge of solid or liquid waste products containing pollutants onto the land surface, or into the aquatic habitats. The wastes that contribute towards water pollution may be broadly grouped into: domestic, industrial, and agricultural types. Sewage is literally the contents of sewers and these comprise the sewerage system that carries the water-borne waster of communities (Becher and Bjoresth, 1987) and (Seiki et al., 1991). Pollutants can -be categorized as four types: nontoxic, toxic, thermal and radioactive, although overlap between theses broad categories is of course possible. Toxic pollutants are metabolic poisons that can seriously injure or destroy the photosynthetic organisms upon which the food chain depends (Doudoroff and Katz, 1953).

An additional environmental problem, which has increased during recent years, is the soluble compounds that have been released from the antifouling paints that contained usually very toxic compounds. These in turn cause damage to living organisms in aquatic environments. Usage of antifouling paints differs regionally according

to legislation, location of the manufacturer, marketing and consumer preferences. Whilst the list of potential booster biocides provided above is substantial, not all compounds are marketed. For example in the UK, although recent legislative changes have occurred, during the last decade usage of antifouling agents was massively dominated by copper oxide followed by (in order of usage) diuron, Irgarol 1051, zinc pyrithioone and dichlofluanid (Environment Agency,1998).

Irgarol 1051 (2-(tert-butylamino)-4-(cyclopropylamino)-6-(methylthio)-1,3,5-triazine) is a biocide widely used in antifouling paint on ships. The s-triazine herbicide Irgarol 1051 is now widely distributed through European coastal waters. Irgarol 1051 has also been showed to be very toxic to growth of fresh water and marin microalgae (Ciba-Geigy, 1995; Dahl and Blanck, 1996), and to the early growth of macroalgae zoospores, (Scarlett et al., 1997).

The sensitivity and response of microalgae to organotin compounds varies from species to species according to shapes and size of the cell wall composition may have different uptake capacities as well as different enzymes for their degradation, so some species appear to be resistant to organotin compounds and possess the ability to accumulate and or degrade these compounds (Tsang et al., 1999). However, very little information is available on uptake of Irgarol and sea nine uptakes and degradation by microalgae. The use of triazine herbicides, including Irgarol 1051, poses environmental risks in the coastal waters due to cause suppression of algal growth by destroying of photosystem II (PSII) (Yang et al., 2019).

Irgarol is widely distributed in coastal water, seawater samples were taken from 26 locations in Singapore measuring TBT, TPhT, and Irgarol. Irgarol was found in 13 samples, (Basheer et al., 2002). . High Irgarol 1051 concentrations were found in Korean sediment samples from shipping and shipbuilding areas. 40 percent of measurements in bays and 20 percent of samples from harbours exceeded the Environmental Risk Limit for sediment set by the Dutch National Institute for Public Health at 1.4 ng/g. Overall, Irgarol 1051 was detected,(Kim et al., 2015). (IMO, 2018) Marine plants appear particularly vulnerable to many of these biocides. The first published study on the herbicidal properties of the booster biocides was by Dahl and Blanck,(1996) on the toxicity of Irgarol 1051 to periphyton communities. In recent years, the toxic organic materials that are released into the environment as a consequence of human activity especially antifouling compounds have had a growing impact on coastal ecosystems. Longterm effects were detected at 0.25 to 1 nM (63 to 250 ng L-l), which is within the range of concentrations reported for coastal waters. Previously used antifoulants, such as TBT and heavy metals, all have broad negative impacts on most marine organisms, and could also be concentrated and transferred across the food chain., (Wang et al., 2020).

The widespread application of antifouling compounds give rise to contamination of marine and fresh water environments. Microorganisms including algae has been used as powerful tools to assess in vitro the toxicity of several environmental pollutants. So, the aim of this work will be: the metabolic response of the marine unicellular alga Chlorella salina to toxicity of the antifouling agent Irgarol 1051 .This work was concentrated on the effect of the biocide antifouling agent Irgarol 1051 on growth and content of some important metabolites: proteins (soluble, insoluble and total) , carbohydrates (soluble , insoluble and total) , amino acids (free, conjugated and total) , fatty acids (saturated , mono and poly-unsaturated) of the marine alga Chlorella salina that usually used for fish feeding.

MATERIALS AND METHODS:

The biological materials chosen in this thesis were the axenic unicellular green alga Chlorella salina obtained from culture collection Botany Department, Faculty of Science, Alexandria University. The basal medium was used in this work described by Boussiba et al.,(1987). The growth of the investigated algae was determined every couple day by cell count using the hemacytometer slide.

The herbicide (Irgarol 1051) was purchased from Fluka company, Cairo, Egypt. The stock solution 1000 mg of the standard Irgarol was prepared in acetone and kept in dark at 4°C. Dilution of this stock solution was mixed with the medium. Different concentrations were prepared and added each to one liter medium. EC50 obtained was 0.50 µg/L. Chosen of two other concentrations (lower and higher) to measure the different parameters for Chlorella salina.

1- Carbohydrates content were estimated according to the method described by Dubois et al.,(1959).
2- 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 at., (1951).
3- The free and total individual amino acids were extracted by the method described by Speckman, et at., (1958).
4- Preparation of fatty acids methyl ester was performed according to the procedure followed by Radwan, (1978).

STATISTICAL ANALYSIS

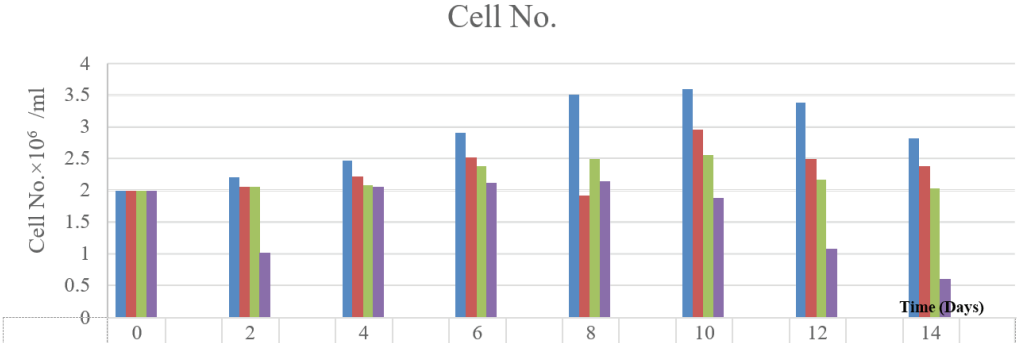
The obtained data were analyzed statistically using two ways ANOVA (Analysis of variance). The difference between means probability levels were analyzed using Duncan's New Multiple Range Test (P< 0.05). F- test were also analyzed (LSD) the least significant difference at 0.05.

RESULT AND DISCUSSION

This work will be concentrated on the effect of the biocide antifouling agent, Irgarol 1051, on growth and content of some important metabolites proteins (soluble, insoluble and total), carbohydrates (soluble, insoluble and total), amino acids (free, conjugated and total) and fatty acids (saturated, mono and poly- unsaturated) of marine algae Chlorella salina that usually used for fish feeding .

Under the effect of concentrations (0.25, 0.50 and 0.75 µg/L) the tested organism remained alive but with different rates of growth. The results cleared also that, the effective concentration (EC50) of Irgarol for Chlorella salina was recorded nearly in concentration 0.5 µg/L at the 8th day.

The data obtained cleared that, 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) and Singla and Garg,(2005). However, Gatidou et al., (2003), found that Irgarol 1051 inhibit growth of Dunaliella tertiolecta at concentration higher than 0.8µg/l and at concentration 3.0µg/l, the compound killed almost all the cells. The result obtained goes with harmony with those obtained by Kaamoush and El Agawany, (2021) who observed that Irgarol inhibit growth in Dunaliella salina at concentrations 0.012 µg/L. The stress effect of booster biocides on growth of algae may be due to the metals found in this compound which cause inhibition of normal cell division (Fisher and Jones ,(1981).Also, Visyiki and Rachlin, (1991) speculated that, inhibition of cell division and cessation of new daughter cells could be due to binding the metals to sulfhydryle groups which are important in regulation cell division. Khalaf et al., (2007), observed that, there was significantly decreased in cell number and dry weight after 7 days, the growth of Chlorella vulgaris towards increasing concentrations of diuron, the dry weight of C. vulgaris after seven days of 0.1 and 0.5 µm diuron treatment was lowered by 2.5 and 5.5 fold, respectively in comparison to control.



	0		2		4		6		8		10		12		14	
Control	2		2.2		2.46	0	2.9	0	3.5		3.6		3.38	0	2.82	0
0.25 µg/l	2	0	2.06	0	2.22		2.52	0	1.92	0	2.96		2.5		2.38	
0.50 µg/l	2	0	2.06	0	2.08	0	2.38	0	2.5	0	2.56		2.16	0	2.02	0
0.75 µg/l	2	0	1.02	0	2.06	0	2.12	0	2.14	0	1.88		1.08	0	0.6	0

Figure (1): Effect of different concentrations of Irgarol 1051 (0.25 , 0.50 and 0.75 µg/l) on growth of Chlorella salina cultured for 14 days

Carbohydrates contents

Results obtained from the experiments 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 Chlorella 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. Khalaf et al., (2007), reported that, there was significant decrease in carbohydrate contents (total & soluble) of Chlorella with increasing dose of antifouling diuron.

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. Kaamoush and El Agawany, (2021) reported that, there was supression in carbohydrate content in Dunaliella salina in culture containing 0.012, 0.025 and 0.050 µg/L of Irgarol. 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. 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 Prado 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 in the two tested algae depended mainly on the concentration of the stress compound and the length of culturing period. The decrease in carbohydrates content may indicate that, the efficiency of photosynthesis began to decrease owing to the destruction of chloroplast pigments (Adjei-Twun and Spliusloesser, 1976).It is worth noticing that, a change in carbohydrates content in the advent of the stationary phase showed nearly parallel similarity to the growth rate of the tested two organisms (El- Mostafa, 1998).

The exposure of microalgae to toxic antifoulings may cause serious physiological alterations that can be readily observed via changes in proteins, carbohydrates, pigment and biomass production of Scenedesmus quadricauda, that's because inhibition of antifouling compound to chlorophyll as well as inhibition of photosynthesis activities which affect production of carbohydrates and other metabolites, Chia et al., (2015).

Table (1): Content of carbohydrate fractions (soluble , insoluble and total in mg/l) in Chlorella salina under the effect of different concentrations of Irgarol 1051 (0.25 , 0.50 and 0.75 µg/l).

Time (Days)	Parameter	Control	Irgarol 1051 concentrations (µg/l)			F (p)	LSD
			0.25 µg/l	0.50 µg/l	0.75 µg/l		
0	Soluble	8.21 ±0.023^{a}	8.21±0.023^{a}	8.21±0.023^{a}	8.21±0.023^{a}	547295.70** (<0.001)	0.002
	Insoluble	18.53±0.035^{a}	18.53±0.035^{a}	18.53±0.035^{a}	18.53±0.035^{a}	4591126.6** (<0.001)	0.002
	Total	*26.74*	*26.74*	*26.74*	*26.74*		
4	Soluble	13.23±0.058^{a}	17.90±0.013^{b}	12.62±0.023^{c}	10.15±0.015^{d}	8971030.1** (<0.001)	0.003
	Insoluble	31.26±0.053^{a}	20.25±0.005^{b}	17.51±0.017^{c}	16.85±0.029^{d}	52187348** (<0.001)	0.003
	Total	*44.49*	*38.15*	*30.13*	*27.00*		
8	Soluble	32.30±0.064^{a}	29.95±0.017^{b}	20.90±0.019^{c}	16.21±0.035^{d}	66316109** (<0.001)	0.002
	Insoluble	30.62±0.063^{a}	19.95±0.001^{b}	17.15±0.014^{c}	13.40±0.012^{d}	81673874** (<0.001)	0.004
	Total	*62.92*	*49.90*	*38.00*	*29.61*		
12	Soluble	29.13±0.012^{a}	28.21±0.012^{b}	19.75±0.002^{c}	16.15±0.071^{d}	4410864** (<0.001)	0.003
	Insoluble	33.21±0.081^{a}	18.21±0.005^{b}	15.82±0.058^{c}	13.25±0.018^{d}	2.46×10^{8}** (<0.001)	0.002
	Total	*62.34*	*46.42*	*36.57*	*29.40*		
16	Soluble	19.74±0.026^{a}	19.05±0.015^{b}	20.54±0.012^{c}	12.26±0.012^{d}	434608.82** (<0.001)	0.003
	Insoluble	32.62±0.060^{a}	21.90±0.005^{b}	10.72±0.017^{c}	8.51±0.033^{d}	7210688.6** (<0.001)	0.002
	Total	*52.63*	*40.95*	*31.26*	*20.77*		

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

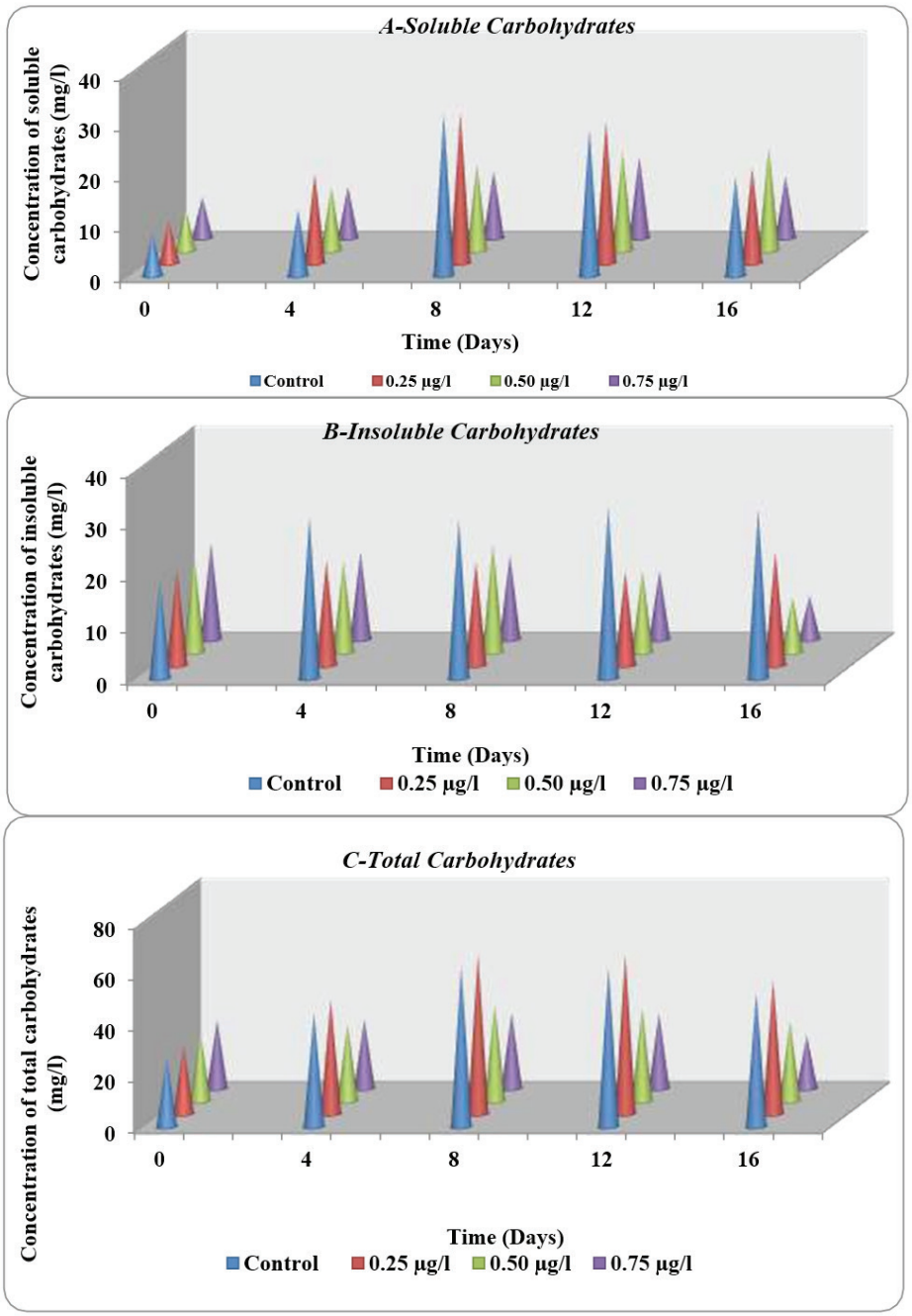


Figure (2): Content of carbohydrate fractions (soluble , insoluble and total in mg/l) in Chlorella salina under the effect of different concentrations of Irgarol 1051 (0.25 , 0.50 and 0.75 µg/l).

Protein

Taking into consideration, the effect of the antifouling agent Irgarol 1051 on content of protein fractions in Chlorella salina after 4, 8, 12, 16 days of culturing, the results that recorded in table (2) and graphed in figure (3) highlights that, at concentrations 0.25 , 0.50 and 0.75 µg/L Irgarol 1051 the content of total proteins increased nearly till the 8th day of culturing then began to decrease reaching minimum at the 16th day of culturing. Irgarol block conversion of excitation energy into chemical energy (Jones,2005 and Sheikh et al., 2009). Total protein contents of the investigated species under normal conditions, as revealed from our data, showed that, Chlorella salina tends to has high levels of proteins. This might be attributed to the fact that, most of algal species have similar physiological functions which are related to either biosynthesis or biodegradation of the same protein macromolecules (Ahmed, 2010).

Numerous species of microalgae such as Spirulina, Chlorella and seaweed including Laminariasp. and Ulva sp. can be considered as source of protein to the diets of cattle, poultry, sheep and rabbits due to high protein content (Holman & Malau-Aduli, 2013).

Dhargalter,(1986) concluded that, the change of biochemical composition of some green algae may be related to the chemical or morphological changes associated with the various metabolic process of the algae. This conclusion seems to explain the different changes of the total amount of proteins content of Chlorella salina in the present investigation. It is also clear from the results that, protein content increased

gradually by increasing period of culturing. However, at the 16th day of culturing, the content of total proteins began to decrease. 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., 1980) and/or to decrease in the rate of protein synthesis (Vaodia and Waisal, 1967). Tam and Wong,(1995) stated that, sever depletion of nutrient supply might be lead to progressive cell death as time proceeded at the end of the experiment. It must be mentioned that, the ability of Irgarol degradation was considered to be species dependant 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).

The ability of some microorganisms to resist toxicity of antifouling agent may be due to the fact that, some microorganisms could prevent the entry of booster biocides into their cells by excreting sorbent to the cellular surface to biosorb this compounds, (Gadd et al., 1990). Luan et al.,(2006), found that, alginate immobilize Chlorella vulgaris was able to continuously remove and degrade the booster biocides even at the highest contamination levels. Also, Exss-Sonne et al. (2000) recorded that the tolerance of micro algae to stress conditions could be elaborated through synthesis or accumulation of new protein. El Agawany et al., (2021) suggested a highly recommendation that the behavior of algae, rich in protein, should be investigated in response to different environmental pollutants.

Table (2): Content of protein fractions in Chlorella salina cultured for 16 days under the effect of different concentrations Irgarol 1051 (0.25 , 0.50 and 0.75 µg/l).

Time (Days)	Parameter	Control	Irgarol 1051 concentrations (µg/l)			F (p)	LSD
			0.25 µg/l	0.50 µg/l	0.75 µg/l		
0	Soluble	17.820±0.250^{a}	17.820±0.250^{a}	17.820±0.250^{a}	17.820±0.250^{a}	80899.416** (<0.001)	0.002
	Insoluble	12.410±0.150	12.410±0.150^{b}	12.410±0.150^{c}	12.410±0.150^{d}	66591.709** (<0.001)	0.002
	Total	*30.230*	*30.230*	*30.230*	*30.230*		
4	Soluble	25.970±1.620^{a}	26.210±1.720^{b}	28.620±2.000^{c}	28.820±1.410^{d}	19788705* (<0.001)	0.003
	Insoluble	32.850±0.710^{a}	30.420±0.410^{b}	25.430±0.050^{c}	22.310±0.210^{d}	23529446** (<0.001)	0.003
	Total	*58.820*	*56.630*	*54.060*	*51.130*		
8	Soluble	32.720±2.000^{a}	29.350±0.820^{b}	28.310±1.540^{c}	25.710±0.720^{d}	30039208** (<0.001)	0.003
	Insoluble	45.510±2.000^{a}	44.200±0.060^{b}	26.160±1.020^{c}	20.420±0.560^{d}	42989758** (<0.001)	0.003
	Total	*78.230*	*73.550*	*54.470*	*46.130*		
12	Soluble	50.640±1.430^{a}	45.120±0.720^{b}	30.150±1.320^{c}	28.350±2.510^{d}	42989758** (<0.001)	0.003
	Insoluble	82.530±0.920^{a}	38.030±0.420^{b}	24.270±0.46^{c}	17.240±1.430^{d}	52066091** (<0.001)	0.004
	Total	*133.17*	*83.150*	*54.420*	*45.590*		
16	Soluble	60.210±2.000	36.410±0.640^{b}	30.240±0.420^{c}	25.760±1.510^{d}	189355.03** (<0.001)	0.002
	Insoluble	54.470±2.240^{a}	42.230±0.820^{b}	22.500±0.670^{c}	14.150±1.200^{d}	42666.09** (<0.001)	0.002
	Total	*114.68*	*78.640*	*52.740*	*39.910*		

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

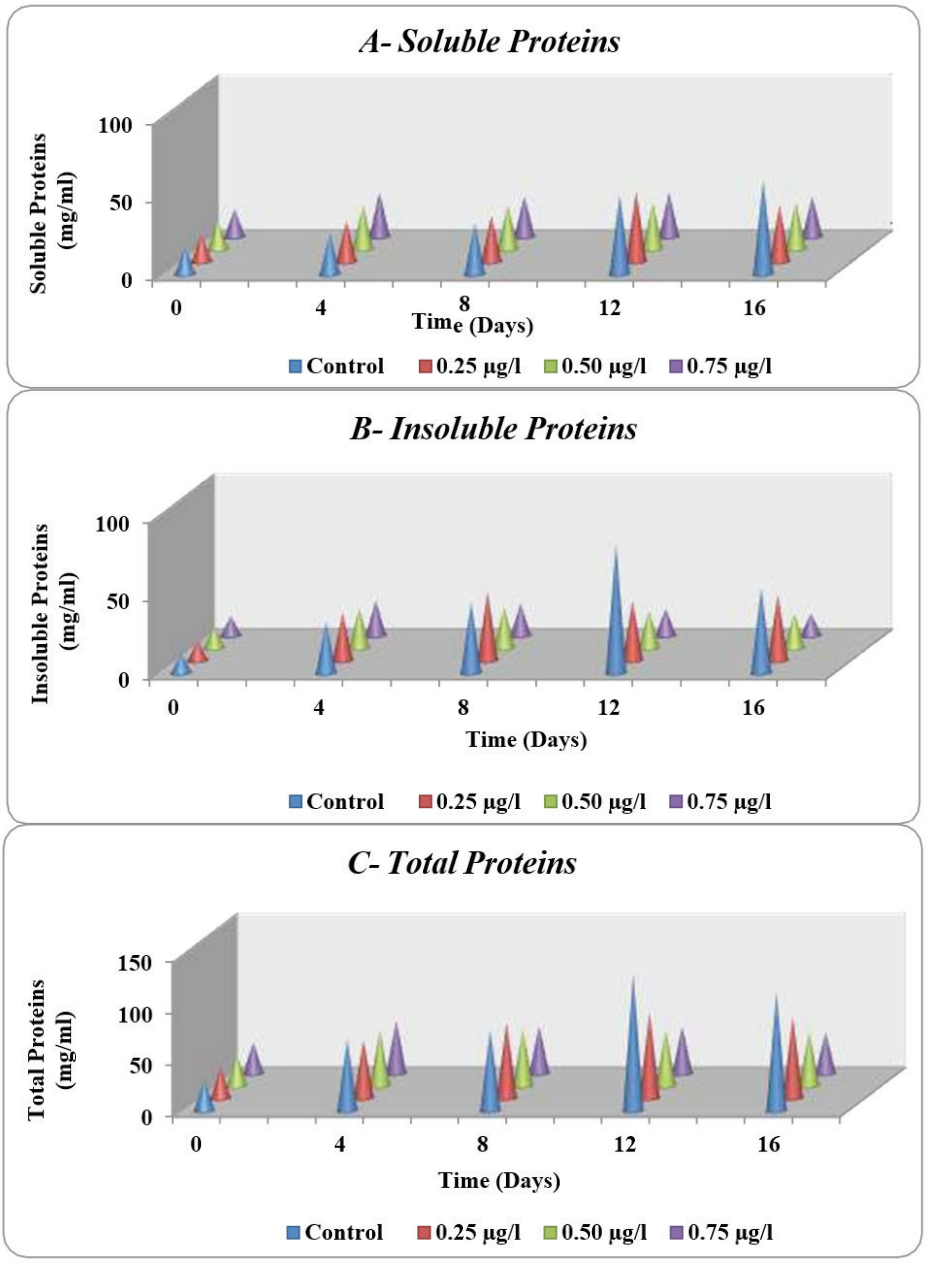


Figure (3): Effect of different concentrations of Irgarol 1051 (0.75 , 0.50 and 0.25 µg/l) on protein fractions content of Chlorella salina cultured for 16 days.

Amino acids

The results obtained for the analysis of free, conjugated and total amino acids for Chlorella salina cleared that, the amino acids of Krebs cycle family (glutamate and aspartate families) surpassed the other families of amino acids. The two families represented nearly 62.34% of the total amino acids in Chlorella salina. The total free amino acids increased gradually under the stress of all the studied concentrations of Irgarol when compared to control. However, Fernandes and Meena,(2007) studied the effect of TBT (antifouling agent) on amino acids of a marine bacteria. They found that, amino acids concentrations decreased with the increase the concentration of the antifouling agent. This idea coincides with our results where total amino acids decreased gradually by increasing the concentration of the antifouling agent. This idea was explained by Song and Huang, (2000), who cleared those antifouling agents like TBT and Irgarol have an inhibitory effect on nitrate reductase activity and consequently a nitrogen metabolism in algae is changed and the equilibrium of nitrogen metabolism destroyed which might influence the nitrogen cycle of the ecosystem. Amino acids

were found to be superior to algal extract. Amino acids are very important in plant growth, development and metabolite synthesis, since they are the basic building blocks of proteins, the synthesis of amino acids in plants is very energy consuming In the light of the experimental results on the effect of different concentrations of Irgarol on the content of essential amino acids, it is clear that, total essential amino acids in untreated cultures represented nearly 3.94% of the total free amino acids for Chlorella salina, while in the conjugated amino acids they represented 96.02 % of the total conjugated ones for Chlorella salina. At the same time, the total amino acids represented nearly 39.37% of the total amino acids. Owing to the fact that nutritional value of protein depends mainly on proportion and availability of its constituents of essential amino acids,), this idea goes in harmony with those obtained by (Brown and Jeffery, 1995). Also, Khalaf et al., (2007) reported that, Proline and total amino acids contents mL-1 of Chlorella vulgarids suspension were significantly decreased with increasing diuron doses to those of the control. Also, Wenqiu et al., (2021) observed that significant decrease in amino acids content obtained in culture of containing different concentrations of copper which used as antifoulant, which suggested that copper materials inhibit biosynthesis of total amino acids.

Xiangyuan, (2012) reported that Irgarol 1051 is toxic to Synechococcus sp. it stimulated cyanobacterial growth, increased the soluble protein content, and enhanced the catalase (CAT) activity at low concentrations, but inhibited them at high concentrations, it may be due to protolysis of insoluble protein.

Table (3): Content of free , conjugated and total amino acid fractions (mg/100mg protein) of Chlorella salina cells cultured for 8 days under different concentrations of Irgarol 1051 (0.25 , 0.50 and 0.75 µg/l).

Amino acids			Type of amino acid	Control			0.25µg/l			0.50µg/l			0.75µg/l		
				Free	Conjugated	Total	Free	Conjugated	Total	Free	Conjugated	Total	Free	Conjugated	Total
Krebs cycle family	Glutamate family	Glutamic	Acidic	0.96	15.09	16.05	0.86	9.38	10.24	1.38	14.57	15.95	1.34	10.47	11.81
		Arginine*	Basic	0.08	4.42	4.50	0.18	5.14	5.32	0.15	4.45	4.60	0.15	2.99	3.14
		Proline	Secondary a.a.	0.61	11.38	11.99	0.64	6.98	7.62	1.05	10.00	11.05	0.92	6.64	7.56
		Histidine*	Basic	0.12	1.64	1.76	0.09	1.86	1.95	0.08	1.17	1.25	0.05	2.52	2.57
		Sub total		**1.77**	**32.53**	**34.30**	**1.77**	**23.36**	**25.13**	**2.66**	**30.19**	**32.85**	**2.46**	**22.62**	**25.08**
	Aspartate family	Aspartic	Acidic	0.35	8.64	8.99	0.39	9.95	10.34	0.34	8.80	9.14	0.35	9.01	9.36
		Threonine*	Aliphatic	0.20	3.79	3.99	0.09	4.47	4.56	0.15	3.67	3.82	0.16	5.06	5.22
		Lysine*	Basic	0.15	4.42	4.57	0.21	5.93	6.14	0.16	4.15	4.31	0.17	4.29	4.44
		Isoleucine*	Aliphatic	0.11	3.42	3.53	0.11	3.79	3.90	0.10	3.20	3.30	0.11	3.64	3.75
		Methionine*	S. containing	0.27	1.69	1.96	0.20	1.90	2.10	0.18	2.85	3.03	0.31	2.53	2.84
		Sub total		**1.08**	**21.96**	**23.04**	**1.00**	**26.04**	**27.04**	**0.93**	**22.67**	**23.60**	**1.10**	**24.53**	**25.63**
	Total			**2.85**	**54.49**	**57.34**	**2.77**	**49.40**	**52.17**	**3.59**	**52.86**	**56.45**	**3.56**	**47.15**	**50.71**
Triose- pyruvic acids family	Triose- family	Glycine	Aliphatic	0.14	5.21	5.35	0.14	5.23	5.37	0.14	5.02	5.16	0.14	5.07	5.21
		Serine	Aliphatic	0.21	3.11	3.32	0.18	3.71	3.89	0.15	3.29	3.44	0.16	4.19	4.35
		Cysteine	S. containing	0.16	0.11	0.27	0.36	0.32	0.68	0.13	0.17	0.30	0.21	0.14	0.35
		Sub total		**0.51**	**8.43**	**8.94**	**0.68**	**9.26**	**9.94**	**0.42**	**8.48**	**8.90**	**0.51**	**9.40**	**9.91**
	Pyruvate family	Alanine	Aliphatic	0.49	5.88	6.37	0.47	6.30	6.77	0.44	5.77	6.21	0.43	6.58	7.01
		Valine*	Aliphatic	0.09	4.51	4.60	0.10	5.48	5.58	0.08	4.26	4.34	0.08	4.92	5.00
		Leucine*	Aliphatic	0.23	6.36	6.59	0.27	8.24	8.51	0.21	5.87	6.08	0.22	7.13	7.34
		Sub total		**0.81**	**16.75**	**17.56**	**0.84**	**20.02**	**20.86**	**0.73**	**15.90**	**16.63**	**0.73**	**18.63**	**19.36**
	Total			**1.32**	**25.18**	**26.50**	**1.52**	**29.28**	**30.80**	**1.15**	**24.38**	**25.53**	**1.24**	**28.03**	**29.27**
Shikime acid family	Phenyl alanine*		Aromatic	0.19	4.52	4.71	0.18	4.50	4.68	0.18	4.16	4.34	0.19	4.89	5.08
	Tyrosine		Aromatic	0.26	3.17	3.43	0.24	3.33	3.57	0.23	3.12	3.35	0.24	3.53	3.77
	Total			**0.45**	**7.69**	**8.14**	**0.42**	**7.83**	**8.25**	**0.41**	**7.28**	**7.69**	**0.43**	**8.42**	**8.85**
Grand total				*4.62*	*87.36*	*91.98*	*4.71*	*86.51*	*91.22*	*5.15*	*84.52*	*89.67*	*5.23*	*83.60*	*88.83*

*Represents essential amino acids.

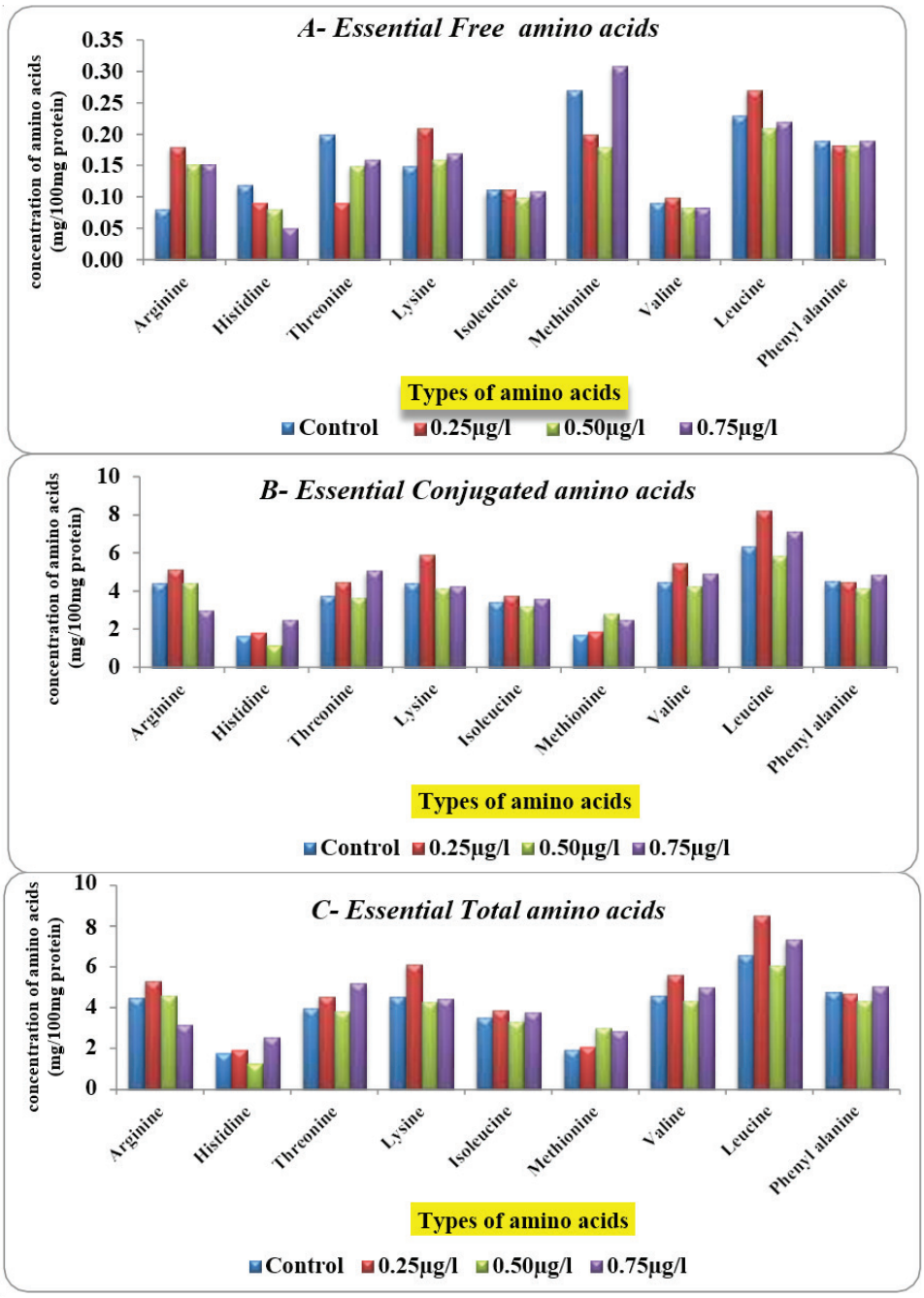


Figure (4): Content of free (A) , conjugated (B) and total (C) essential amino acids fractions of Chlorella salina cells cultured for 8 days at different concentrations of Irgarol 1051.

Fatty acids

It is well known fact that, lipid production including fatty acids usually differed between genera species and strains of micro-organisms, (Johansen et al., 1987). These results coincide with those obtained in our work where Dunaliella salina was found to be more sensitive to Irgarol than Chlorella salina. However, total lipid fractions in healthy phytoplankton vary substantially from less than 1% to more than 40% of dry weight (Dubinsky et al., 1978).The stress response was more prominent in mono-unsaturated fatty acids than in the other two groups of fatty acids and the more the increase in the concentration of Irgarol, the more the stress effect and consequently the more the decrease in the content of total fatty acids.

Environmental factors were found by many authors to affect the proportions of fatty acids. Also, Simonopoulos,(1991) found that, microalgae were a good source of Omega-3 fatty acids which are

protective factors against chronic diseases, diabetes and cancer. Chu and Dupuy, (1980) concluded that, the changes in the relative amount of poly-unsaturated fatty acids may be attributed to effects on the destruction bath way of fatty acids. Xu et al., (1997&1998) reported that, the reduction of the poly-unsaturated fatty acids fractions might be due to reduction in membrane fluidity and permeability. These results go in harmony with our results where some poly-unsaturated fatty acids specially those belonging to Omega-3 disappeared completely at higher concentrations (0.75µg/l Irgarol) in Chlorella salina, while at concentration 0.025µg/l only one fatty acids (archidonic acid) disappeared. Our results are in agreement with those obtained by Soizic et al.,(2021), who observed that Diuron and irgarol are photosynthetic inhibitors and they cause inhibition of fatty acids synthesis.

Saturated fatty acids are the only group of fatty acids that increased under the stress of the three tested concentrations of Irgarol in the studied organism. Dowidar, (1983) mentioned that, saturated fatty acids were more dominant than unsaturated ones under stress conditions. The same conclusion was also reported in our results. In our laboratory it was found that, the maximum values of fatty acids usually attained at the end of the log or at the beginning of the stationary phases. At these two phases, the nutrient values of culture medium usually decrease. So, we usually analyze the fatty acids in the tested organisms cultured for 12 days. These results coincide with those obtained by El-Maghrabi,(2002) and Al-Osaimi, (2010).

Environmental factors can affect both the relative proportions of fatty acids (Fisher & Schwarzenbach, 1978) as well as, the total amount of lipids (Conover, 1975). This idea coincide with those obtained in our work where the total fatty acids in theorganism decreased by increasing the concentration of Irgarol.

Table (4) :Effect of different concentrations of Irgarol 1051 on fatty acids content of Chlorella salina cultured for 12 days .

Fatty acids		Control	Irgarol 1051 concentration		
			0.25 µg/l	0.50 µg/l	0.75 µg/l
Saturated	C 14:0 Myristic	1.021	1.708	1.751	1.481
	C 16:0 Palmitic	2.724	2.138	2.129	2.429
	C 18:0 Stearic	0.016	---	---	---
	C 20:0 Arachidic	0.193	0.194	0.205	0.147
	Total	3.954	4.040	4.085	4.057
	% of increasing (+) or decreasing (-)	100%	(+) 2.18%	(+) 3.31%	(+) 2.61%
Mono -un saturated	C 18:1 Oleic	6.232	2.722	1.011	0.113
	C 16:1 Palmitoleic	0.451	0.365	0.300	0.040
	C 22:1 Erucic	0.158	0.132	0.102	---
	Total	6.841	3.219	1.413	0.153
	% of increasing (+) or decreasing (-)	100%	(-) 52.95%	(-) 79.35%	(-) 97.76%
Poly- un saturated	C 18:2 Linoleic	0.221	0.055	0.041	0.025
	C 18:3 Linolenic	4.621	4.293	2.450	0.631
	C 20:4 Archidonic	6.072	6.036	3.480	---
	C20:5Eicosa-pentaenoic	1.325	0.630	0.243	---
	*C22:*6Docosa-hexaenoic	2.490	2.250	1.049	0.419
	Total	14.729	13.264	7.263	1.075
	% of increasing (+) or decreasing (-)	100%	(-) 9.95%	(-) 50.69%	(-) 92.70%
Total fatty acids		25.524	20.523	12.761	5.285
% of increasing (+) or decreasing (-)		*100 %*	*(-)19.59%*	*(-)50.00%*	*(-)79.29%*

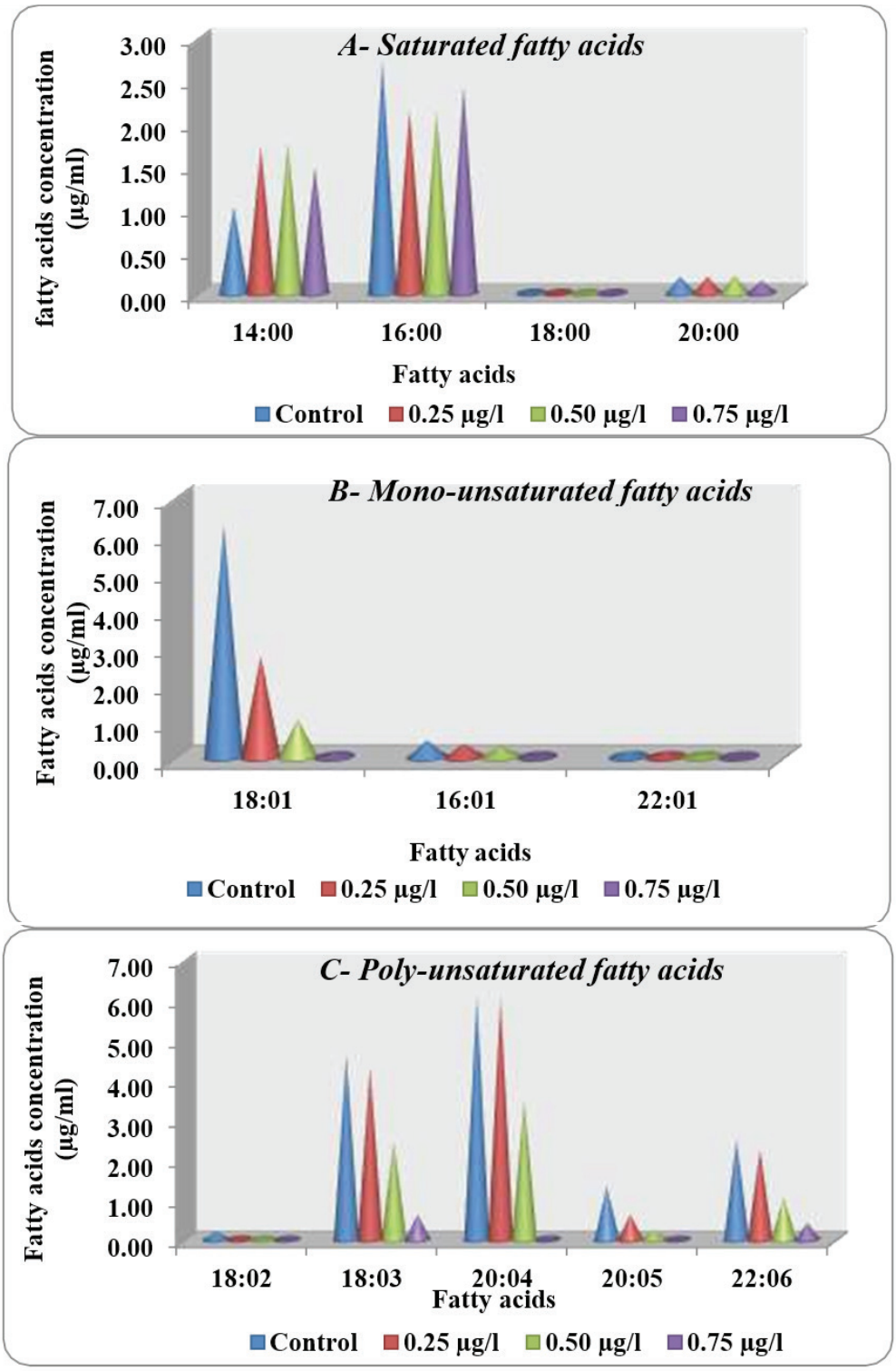


Figure (5): Effect of different concentrations of Irgarol 1051 on fatty acids content of Chlorella salina cultured for 12 days.

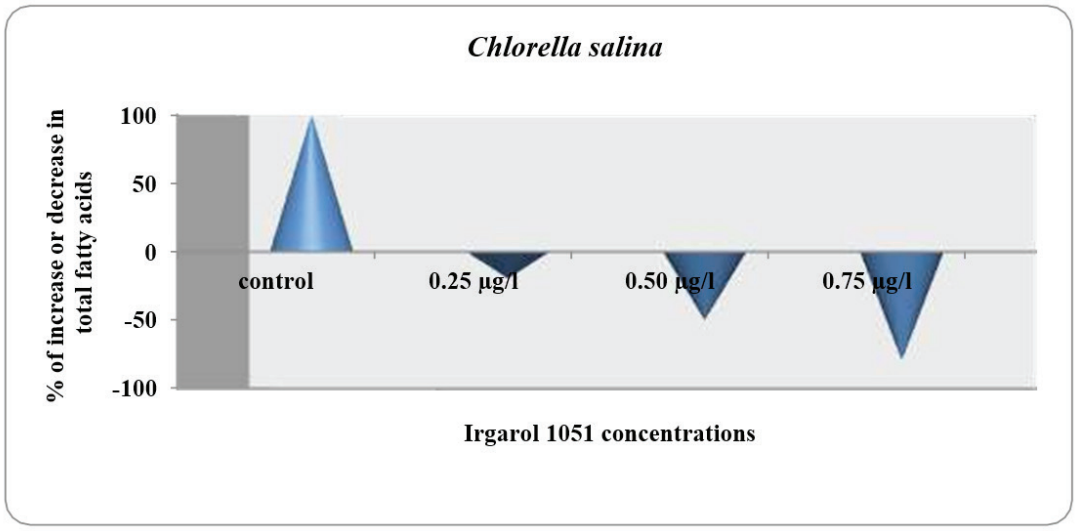


Figure (6): Percent of increase (+) or decrease (-) in total fatty acids under the effect of different concentrations of Irgarol 1051 in Chlorella salina cultured for 8 days .

CONCLUSION

Irgarol 1051 (2-(tert-butylamino)-4-(cyclopropylamino)-6-(methylthio)-1,3,5-triazine) is a biocide widely used in antifouling paint on ships to prevent fouling growth. It is used booster herbicides in antifoulants and may exert potent toxic effects on marine primary producers such as microalgae, where majority of the toxicity data are based on growth inhibition of essential metabolites such as proteins, carbohydrates, amino acids and fatty acids. The results cleared also that, the effective concentration (EC50) of Irgarol for Chlorella salina was recorded nearly in concentration 0.5 µg/L at the 8th day. The data obtained cleared that, 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. all the tested concentrations of Irgarol were inhibitor to carbohydrates content of Chlorella salina. At concentrations 0.25 , 0.50 and 0.75 µg/L Irgarol 1051 the content of total proteins increased nearly till the 8th day of culturing then began to decrease reaching minimum at the 16th day of culturing. The stress response was more prominent in mono-unsaturated fatty acids than in the other two groups of fatty acids and the more the increase in the concentration of Irgarol, the more the stress effect and consequently the more the decrease in the content of total fatty acids.

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