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Full Length Research Paper

Metabolic response of *Cyclotella meneghiniana* Kutzing to toxicity of the antifouling agent Irgarol 1051

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Soluble toxic compounds that are leached out from antifouling paints cause unpalatable taste and disagreeable odor. This in turn will directly or indirectly affect the metabolic activities of the aquatic biota. In this work, reliable studies about the expected effects of these toxins on living organisms were conducted. Microorganisms especially algae were used as powerful tools to assess *in vitro* metabolic response on several environmental toxins. The obtained results reveal that the antifouling Irgarol 1051 was a strong inhibitor to the studied microalga *Cyclotella meneghiniana*. It became apparent from the laboratory results that water polluted by these leached toxins caused a substantial metabolic inhibition and the degree of inhibition is selectively concentration dependent.

Key words: Antifouling agent, *Cyclotella meneghiniana*, Irgarol 1051, metabolic activities.

INTRODUCTION

Water purity can have a great influence on the ability of plants and animals to exist and grow, which in turn affects human health since it can carry toxins and bacteria, as well as, having unpalatable taste and disagreeable odor (Palmer, 1980). 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 (Seiki et al., 1991). An additional environmental problem which has increased during recent years is due to the soluble active ingredients (biocides) in the paints of ships that have been leached out from the antifouling paints which contained usually very toxic compounds (Hoch, 2001). These in turn cause damage to the metabolism of living organisms in aquatic and land environments. However, some microorganisms have the ability to resist and accumulate these toxins, which in turn will be more serious to consumers whether fishes, animals or humans.

Irgarol 1051 (2-methylthio -4-terbulylamino -6-cyclopropylamino -s-triazine) is used now in aquatic antifouling paints after the restriction of using tributyltin (TBT) as antifouling agent (Zamora-Ley et al., 2006). 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 (Biselli et al., 2000; Lamoree et al., 2002; Thomas et al., 2003). 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; Konstantinou and Albanis, 2004; 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). The toxic effects of Irgarol 1051 against algae, crustaceous and fish have been reported by Van Wezel and Van Vlaardingen (2004). Concentrations of Irgarol 1051 have been found to be highest in and adjacent to marines and fishing harbours range between 0.02 and 0.70 µg/l. Readmann et al. (2004) reported toxic effects

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of Irgarol 1051 at low concentrations of 0.07 ng/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.

This work is concentrated on the response of the microalga *Cyclotella meneghiniana* to differences of the ambient antifouling agent Irgarol 1051 on its growth and content of some important metabolites viz; chlorophyll fractions, protein, protein profile, photosynthesis, respiration and the destructive effects of the total antioxidants.

MATERIALS AND METHODS

The selective biological material is the unicellular microalga *Cyclotella meneghiniana* Kutzing -usually used for fish feeding; obtained from the culture collection of Prof. Khaleafa, Faculty of Science, Alexandria University. The tested microalga was grown in brackish water medium as described by Schlösser (1994). The pH of the culture media was adjusted to 7.5 using pH-meter then sterilized by using the bacterial filter. The tested alga was grown in Erlenmeyer pyres-glass flasks (capacity 250 ml) containing 50 ml culture medium. The cultures were maintained under controlled laboratory conditions (temperature of 22 ± 2°C and light intensity of 80 □mol m^{-2} s^{-1}) in controlled culture chamber for 16 days and under a photoperiod of 16/8 h light/dark cycles. Three independent experiments were performed for each treatment.

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 nearly within the range of those found in marines.

Growth rate was estimated by cell number using haemocytometer slide. The growth rate was calculated by using the formula proposed by Stein (1979). Growth rate (number of cells / day) = (log N_2/N_1) x ($3.322/T_2$-T_1), where N_1 and N_2= number of cells; T_1 and T_2= times in days.

The chlorophylls content were estimated in the acetone extracts by using the dichromatic equations of Jeffrey and Humphrey (1975): Chl. a (µgml^{-1}) = 11.47 E_{664} - 0.40 E_{630}; Chl. c_1+c_2 (□gml^{-1}) = 24.36 E_{630} - 3.73 E_{664}.

Protein was extracted from the algal cells by the method described by Rausch (1981). Protein content and its fractions were estimated by the method described by Hartree (1972). The protein content was calculated from a standard curve made from working standards of similarly treated bovine serum albumin as mgl^{-1} cultures.

Protein profile was performed according to the method described by Laemmli (1970). The freshly cell pellets obtained by centrifugation of 10 ml culture were ground in a mortar in the presence of small volume of 0.5 M Tris-HCl buffer (pH 7.2). The suspension was centrifuged and the supernatant was concentrated in pre-activated dialysis tubing over a sucrose bed. Few drops of glycerol were added to increase viscosity and 2 to 3 drops of bromophenol blue as an electrophoresis marker. Electrophoresis was carried out in 12% acrylamide running gel and a 4% stacking gel, with 0.025 M tris 0.19 M glycine buffer pH 8.3. Further details of the methods used in gel preparation and electrophoresis were described previously by Moore et al. (1980). Electrophoresis was performed at 25°C in a vertical tank apparatus maintained at 100 V using a constant-voltage power supply, until bromophenol blue tracking dye reached the bottom of the gel. After electrophoresis, gels were stained with 2% Coomassie brilliant blue G 250 in water-methanol-acetic acid (4.5:4.5:1) for 4 h at 37°C, after that, they were destained by agitation in the same solvent without dye, and stored in a fixing solution (7% acetic acid) for at least 30 min at 30°C and rinsed with deionized distilled water. The gels were then photographed.

Photosynthesis and respiration

The photosynthetic activity was measured polarographically as oxygen evolution using a Clark type electrode (YSI, model 53). The actinic white light was obtained from a 150 W. tungsten lamp. The test organism was harvested every 4 days intervals till the end of the experiment. Photosynthetic activity was measured as (µmolO_2 evolution/mg chlorophyll/hour). Respiration was measured in the dark and expressed as (O_2 uptake µmolO_2h^{-1}).

Total antioxidants activity

Determination of the total antioxidants activity was performed by the reaction of antioxidants in the sample with a definite amount of exogenously provided hydrogen peroxide as described by Koracevic et al. (2001). The antioxidants in the sample eliminated a certain amount of the provided H_2O_2. The residual H_2O_2 was determined colorimetrically by using the standard package (R1, R2 and R3) supplied from the manufacture's instruction (Biodiagnostic and research reagents Egypt); an enzymatic reaction which involves the conversion of 3, 5, dichloro-2-hydroxy benzensulphonate to a colored product. The absorbance of the blank (AB) and sample (ASA) against distilled water was read immediately at 505 nm. The overall antioxidants capacity was then calculated as mM l^{-1} by using the following formula; mM l^{-1} = A_B - A_{SA} × 3.33 (Comba et al., 2004).

Statistical analysis

The data were the means ± SD of at least three independent experiments. The obtained data were analyzed statistically using the least significant difference (LSD) at 0.05.

RESULTS AND DISCUSSION

The data recorded in Figures 1 and 2 show that suppression in growth parameters (growth rate and chlorophylls content) of *Cyclotella meneghiniana* under the effect of the tested different concentrations of Irgarol 1051 may be due to the increasing toxicity of the biocide. The same results were also obtained by Munnas (2003) and Singla and Garg (2005). The rate of growth depended mainly on the level of irgarol 1051 concentration and length of culture period. This idea is in harmony with those obtained by Gatidou et al. (2003). The stress effect of Irgarol 1051 on growth of *C. meneghiniana* may be due to the toxic metals (biocides) that leached out from the antifouling paint. These cause inhibition of normal cell division due to the binding of the

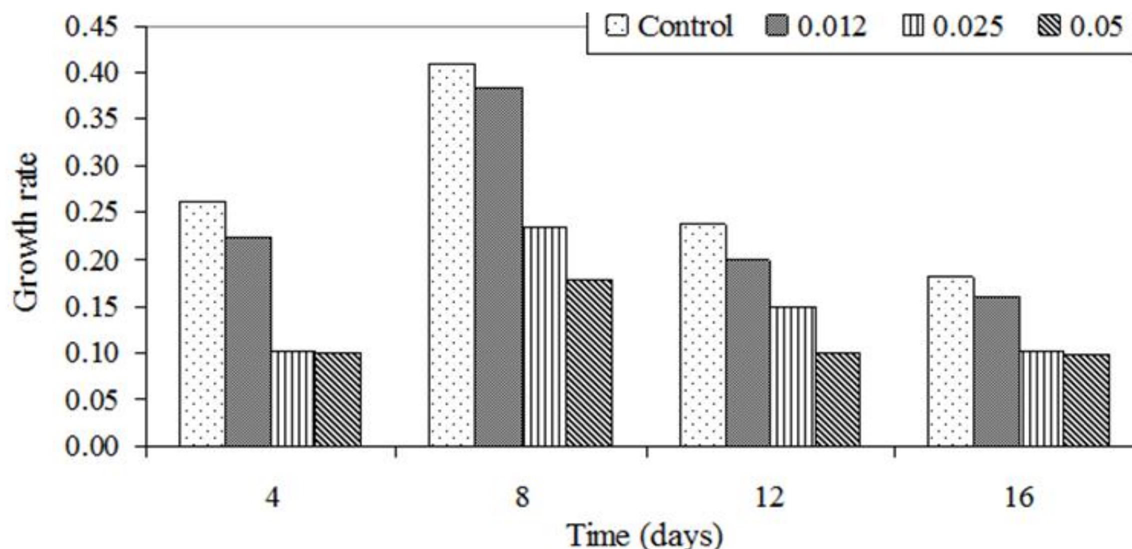

Figure 1. Effect of different concentrations of Irgarol 1051 (μg/l) on growth rate (number of cells/day) of *Cyclotella meneghiniana*.

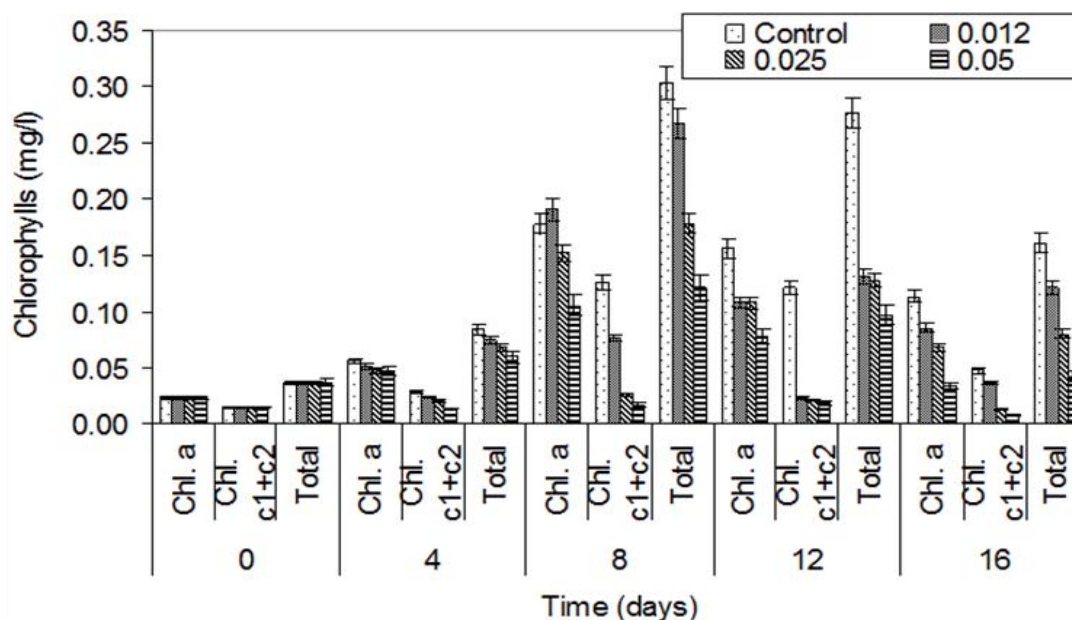

Figure 2. Effect of different concentrations of Irgarol 1051 (μg/l) on chlorophylls content of *Cyclotella meneghiniana*. (Data are mean of three replicates ± SD).

biocides to sulfhydryle groups which are important in the regulation of cell division (Visviki and Rachlin, 1992; Sidharthan et al., 2002). Moradi and Ismail (2007) and Ghezelbash et al. (2008), reported that reduction in chlorophyll content with reduced growth rate was due to decrease in photosynthetic rate. Also, Jones (2005) and Zamora-Ley et al. (2006) reported that the antifouling boostering agent Irgarol 1051 was a strong inhibitor of photosystem II (PSII).

The changes of biochemical composition of some microorganisms under the stress effect of toxins may be due to the block conversion of excitation energy into chemical energy (Jones, 2005; Sheikh et al., 2009). The data recorded in Table 1 shows that protein content increased gradually by increasing the period of culturing. At the end of the experiment (16 days), the content of total proteins decreased. This may be due to decrease in insoluble protein fractions and deficiency of nutrients

Table 1. Effect of different concentrations of Irgarol 1051 (µg/l) on protein fractions content (mg/l) of *Cyclotella meneghiniana*.

Time (days)	Parameter	Control	0.012	0.025	0.050
4	Insoluble	14.221±0.002^{a}	14.456±0.001^{a}	13.752±0.002^{b}	14.135±0.004^{a}
	Soluble	15.226±0.002^{a}	15.516±0.002^{q}	12.234±0.002^{b}	12.012±0.001^{b}
	Total	29.447	29.972	25.956	22.147
8	Insoluble	22.720±0.200^{a}	15.752±0.100^{b}	17.214±0.012^{c}	16.321±0.201^{d}
	Soluble	43.310±0.150^{a}	43.195±0.104^{a}	35.310±0.130^{b}	21.214±0.420^{c}
	Total	66.030	58.947	52.524	37.445
12	Insoluble	30.641±0.143^{a}	20.197±0.013^{b}	25.701±0.205^{c}	18.502±0.104^{d}
	Soluble	48.123±0.031^{a}	40.161±0.022^{b}	30.214±0.001^{c}	20.370±0.108^{d}
	Total	78.764	60.358	55.915	38.872
16	Insoluble	34.210±0.200^{a}	25.241±0.022^{b}	27.520±0.103^{c}	20.210±0.040^{d}
	Soluble	42.420±0.220^{a}	40.021±0.001^{b}	30.120±0.024^{c}	22.341±0.051^{d}
	Total	76.630	65.262	57.640	42.551

Data are mean of three replicates ± SD. Different superscripts are significant (P ≤ 0.05).

Table 2. Effect of different concentrations of Irgarol 1051 (µg/l) on the activity of photosynthesis (µmolO_2 evolution / mg chlorophyll / h) and respiration (O_2 uptake µmolO_2 h^{-1}) of *Cyclotella meneghiniana*.

Time (days)	Parameter	Control	0.012	0.025	0.050
4	Photosynthesis	1.67±0.24^{a}	1.22±0.17^{b}	0.82±0.10^{c}	0.51±0.12^{d}
	Respiration	4.73±0.14^{a}	3.02±0.11^{b}	1.80±0.21^{c}	1.42±0.19^{d}
8	Photosynthesis	1.93±0.22^{a}	1.21±0.15^{b}	0.51±0.18^{c}	0.32±0.21^{d}
	Respiration	3.52±0.18^{a}	2.30±0.10^{b}	1.34±0.18^{c}	1.22±0.27^{d}
12	Photosynthesis	1.71±0.19^{a}	1.20±0.13^{b}	0.42±0.25^{c}	0.22±0.24^{d}
	Respiration	3.16±0.31^{a}	1.64±0.22^{b}	1.24±0.20^{c}	1.04±0.18^{d}
16	Photosynthesis	1.60±0.10^{a}	1.02±0.18^{b}	0.42±0.11^{c}	0.19±0.19^{d}
	Respiration	1.70±0.17^{a}	1.31±0.21^{b}	1.02±0.13^{c}	1.01±0.27^{c}

Data are mean of three replicates ± SD. Different superscripts are significant (P ≤ 0.05).

which increase proteolysis (Cooke et al., 1979, 1980) and/or due to decrease in the rate of protein synthesis (Vaadia and Waisel, 1967; Tam and Wong, 1995). The the toxicity effects of the different levels of Irgarol 1051. The greater the toxic effects of the antifouling agent and the length of culturing period, the greater was the content of soluble proteins and the less was the content of insoluble ones. The decrease in content of insoluble protein may be due to its degradation and/or weak synthesis of the soluble ones. This in turn will cause accumulation of the soluble ones (Salah El-Din, 1994; Ahmed, 2010; Kaamoush 2011).

Data represented in Table 2 shows that the quantity of O_2 evolution in photosynthesis and O_2 uptake in data recorded also cleared that although total protein content decreased under the stress effect of Irgarol 1051, yet protein groups (soluble and insoluble) differed under respiration of *C. meneghiniana* depended mainly on the concentration and length of culturing period. Under these conditions, both photosynthesis and respiration decreased gradually till the end of the experiment. The rate of the stress effect on the activity of these two parameters increased gradually by increasing the concentration of Irgarol 1051. At the 8th day of culturing, the percent of decrease in respiration reached 34.86, 62.78 and 65.34% at the different concentrations of the tested Irgarol 1051, respectively. In the case of photosynthesis, it decreased by 37.31, 73.58 and 83.42%

Table 3. Effect of different concentrations of the antifouling agent Irgarol (1051) on protein profile bands in *Cyclotella meneghiniana*.

Rf	KDa	Control (un-treated)	0.012 µg/l			0.025 µg/l			0.050 µg/l		
			a	b	c	a	b	c	a	B	c
0.0 - 0.1	222 - 201	1	-	-	1	-	-	1	-	-	1
0.1 - 0.2	174 - 139	-	-	-	-	-	-	-	-	-	-
0.2 - 0.3	118 - 92	2	2	-	-	1	-	1	1	-	1
0.3 - 0.4	89 - 64	-	1	1	-	-	-	-	1	1	-
0.4 - 0.5	63 - 48	1	1	-	-	1	-	-	1	-	-
0.5 - 0.6	30 - 27	-	-	-	-	1	1	-	-	-	-
0.6 - 0.7	24 - 22	2	1	-	1	1	-	1	-	-	2
0.7 - 0.8	17 - 14	2	1	-	1	1	-	1	1	-	1
0.8 - 0.9	12 - 9	1	1	-	-	1	-	-	1	-	-
Total		9	7	1	3	6	1	4	5	1	5

a = Present bands; b = newly appeared bands; c = disappeared bands.

respectively. This means that the process of photosynthesis was affected to a larger extent under the toxicity stress of Irgarol 1051 than respiration. Moradi and Ismail (2007) and Ghezelbash et al. (2008) represented that reduction in chlorophyll content with reduced growth rate was due to decrease in photosynthetic rate. Also, Jones (2005), Zamora-Ley et al. (2006) and Kaamoush (2011) reported that the antifouling boostering agent Irgarol 1051 is a strong inhibitor of photosystem II (PSII) with high efficiency (toxicity) to photosynthetic micro-organisms.

Most of the living microorganism during their adaptation to the external stresses changes in the macromolecule content and in the enzyme activities usually occurs (Schiewer and Jonas, 1977; Hagmann and Wittenburg, 1989; Hagemann et al., 1989). Therefore, changes in the protein composition of these organisms may also be involved in adaptation processes. During these processes of adaptations, disappearance and synthesis of stress, induced proteins has been demonstrated in living organism (Hecker et al., 1988; Apte and Bhagwat, 1989; Hagemann et al., 1990). In our work, the gel of the total soluble protein profile of the tested organism at the different concentrations of Irgarol 1051 showed bands distributed through the gels. The number of bands that disappeared, were newly formed and remained compared to the control which depended mainly on the concentration of Irgarol 1051 (Table 3 and Figure 3). The results of Hoyos and Zhang (2000) are in agreement with these results. The results of Salah El-D (1994) confirmed that most of the living organism species have similar physiological fractions which are related to biosynthesis or biodegradation of some macromolecules. This conclusion seems to explain the different changes of the amount of total soluble protein bands in the stressed microorganisms. Exss-Sonne et al. (2000), Hifney (2002) and Ahmed (2010) reported that tolerance of an organism to stress conditions could be achieved through the synthesis or accumulation of new types of protein. The above results were nearly in harmony with our results for *C. meneghiniana*.

From the results obtained concerning the antioxidative defense systems (Figure 4), it is clear that the activity of total antioxidants in *C. meneghiniana* amended with different concentrations of Irgarol 1051 showed a significant progress, if there was any increase through the entire period of the experiment. Careful examination of the results obtained reveal that by increasing concentration of Irgarol 1051, a progressively greater increase in the activity of the total antioxidants was obtained under low and mild stress (Comba et al., 2004). However, Jungklang (2005) reported that in living organisms subjected to high stress, the balance between the production of ROS and quenching activity of the antioxidants in upset, results in antioxidant stress. Our results lend a strong support to the results obtained by Bor et al. (2003), Chaparzadeh et al. (2004) and Jungklang (2005).

Based on the fact that the different environmental stress conditions are likely to affect the same physiological parameters, it is not really surprising to find that the present state of Irgarol 1051 stress triggers the expression of the antioxidative defense systems. This is evident from the fact that total antioxidants which included non-enzymatic and enzymatic antioxidants, showed in general an increase with increasing stress indicating the cell capacity to overcome the stress. The conclusion can be substantiated if the degree of stress is not so high to concentration limit that may cause damage and death of the organism. In this work, the same conclusion was also observed for *C. meneghiniana* where the total antioxidant activity began to decrease at the higher concentration of Irgarol 1051.

In conclusion, this work clears that waters polluted by these booster biocides of Irgarol 1051 which leached out

Figure 3. Electropherogram showing the scanning results of protein profile bands in *C. meneghiniana* cells asthe control and at the effect of different concentrations of Irgarol 1051. **(A)** 0.012 µg/l. **(B)** 0.025 µg/l. **(C)** 0.050 µg/l.

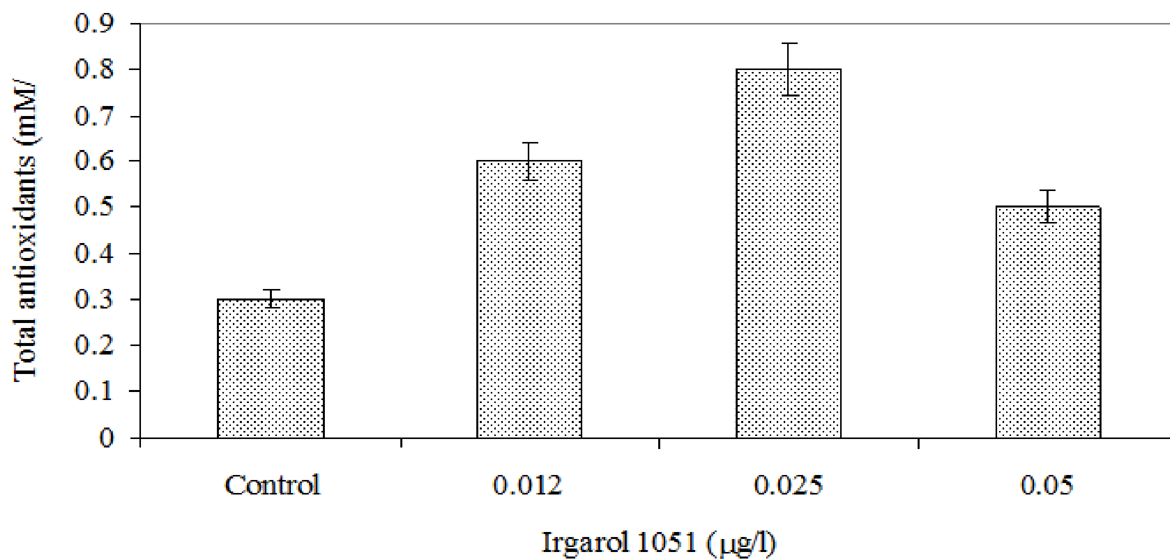

Figure 4. Total antioxidant capacity (mM/l) of *C. meneghiniana* at control and at the three tested Irgarol 1051 concentrations. (Data are mean of three replicates ± SD).

from the antifouling paints will cause weak growth, damage or death of the periphyton communities.

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