Laboratory Study of Stimulatory or Inhibitory Effects of Three Heavy Metals Ni, Zn And Cu on Growth of Marine Unicellular Green Alga *Dunaliella tertiolecta* Used as Biological Indicator for Monitoring and Protecting Aquatic Environment

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Abstract:

This study focused on heavy metal pollution as one of the most dangerous type of pollutants. The presence of heavy metal ions in water can create a serious damage to the aquatic life because they are accumulated through the food chain and produce toxic effects in plants, animals and human. Port operations can lead to environmental impacts on air, water and land. Many communities with environmental justice concerns also experience disparities in health outcomes that they attribute to exposure to emissions from port operations. The biomarker concept has been associated with an 'early warning' of pollution-induced stress. The work designed to estimate the stimulatory or inhibitory effects of three heavy metals (nickel, zinc and copper) on the growth of the marine unicellular green alga *Dunaliella tertiolecta* which used as biological biomarker for different pollutants. Three heavy metals were selected during the present study based on their abundance in the industrial waste water around Alexandria, as well as, their effect on the receiving aquatic ecosystems, also they considered essential micro-element for growth of different algal species. The results proved that Ni^{2+} were less toxic than both Zn^{2+} and Cu^{2+}, while Cu^{2+} is the most toxic one on growth of *Dunaliella tertiolecta*. Stimulatory effect of the three heavy metals obtained at low concentrations only but increasing of heavy metal concentration led to cause inhibitory effect but with different degrees depending on metal type and concentration used.

Key words: Heavy metals, Pollution, Biomarker, *Dunaliella tertiolecta.*

Introduction

The use of the marine environment for waste disposal must only be undertaken after first conducting as rigorous an assessment as possible of the probable impact. The procedures by which this assessment is conducted should be based on a comprehensive scientific assessment of the local environment as well as on forecasting the potential effects that an activity might impose on that environment and human well-being dependent on it. The process by which the decision is taken often centers on a document known by different names - Environmental Impact Report, Environmental Impact Assessment and Environmental Impact Statement. Scientific input to the

process of environmental impact assessment may be required, first when the scope of the investigations is being determined, secondly in the specific investigations required to provide the necessary data and finally, in direct advice to decision-makers in interpreting scientific data and in allaying public concern. Further scientific input is required as follow-up action such as monitoring and review. The main polluted wastes that discharged directly into marine environment are:

a- Nutrients and natural organic materials in the form of suspended solids, ammonia and other natural oxygen-demanding materials.
b- Heavy metals such as lead and cadmium in greater concentrations.
c- Pathogens, like bacteria and viruses.
d- Toxic chemicals which, by affecting the genetic code (genotoxic) may cause carcinogenic, mutagenic and diseases.
(**IMO/FAO/Unesco/WMO/WHO/IAEA/UN/UNEP, 1986).**

Metals with atomic masses between 54.63 and 200.59 and special weights of more than 5 g/cm3 are classified as heavy metals (**Dias *et al*., 2002**). These elements are natural components of earths' crust, which are released into environment because of the natural and human activities. In biology, heavy metals are the atoms with toxic effects such as: Al, As, Br, Cr, Co, Cu, Cd, Fe, Hg, Ni, Mn, Pb, Se and Zn. Volcanoes, burning of forests and aeration of stones and minerals are counted as natural polluting events (**Fomina *et al*., 2005 and Çabuk *et al*., 2005**). Digging and mining processes of metal melting factories, burning of wastes, usage of fossil fuels in companies, utilization of pesticides and other ones could be mentioned as human activities in polluting environment (**Arshad and Shafaat, 1997; Fomina *et al*., 2005;Saber *et al*., 2011**). Moreover, high metal ions concentrations in wastewater can kill microorganisms or at least diminishes their activities during biological treatment of wastewater (**Volesky, 1994**). Heavy metals like Hg, Cr, Pb, Zn, Cu, Ni, As, Cd, Sn, Co are bio-accumulative and non-biodegradable toxins, the presence of such metals in aquatic environments cause severe damage to aquatic life, hence they find their way to the food chain (**Hassan *et al*., 2013 and 2014; Brouersa *et al*., 2016**; **Jelan, 2017**). Heavy metal pollution has resulted in many problems for human health and aquatic ecosystems as well (**Rai *et al*., 1981a** and **Inthorn *et al*., 1996**). Industry represents a potential source of a variety of toxic heavy metals. According to their potential hazard, the heavy metals Pb, Hg, As and Cd were ranked by the U.S. Agency for Toxic Substances and Disease Registry (ATSDR) as first, second, third, and fourth highly toxic metals, respectively (**Howard, 2002**). However, due to the recently discovered extreme acute toxicity of Cd, this metal has now joined Pb and Hg as one of the major three heavy metals greatly hazardous to human health (**Volesky, 1990**).

Many hull cleaning and coating companies claim significant recovery rates for biological material (contaminants such as heavy metals and polymers are not generally mentioned) for both remotely operated cleaning equipment and for diver operated brushes etc. Regulations in the new Convention cover: the design, construction, operation and preparation of ships so as to facilitate

safe and environmentally sound recycling without compromising the safety and operational efficiency of ships; the operation of ship recycling facilities in a safe and environmentally sound manner; and the establishment of an appropriate enforcement mechanism for ship recycling, incorporating certification and reporting requirements. Upon entry into force of the Hong Kong Convention, ships to be sent for recycling will be required to carry an inventory of hazardous materials, which will be specific to each ship. An appendix to the Convention provides a list of hazardous materials the installation or use of which is prohibited or restricted in shipyards, ship repair yards, and ships of Parties to the Convention. Ships will be required to have an initial survey to verify the inventory of hazardous materials, additional surveys during the life of the ship, and a final survey prior to recycling. Ship recycling yards will be required to provide a "Ship Recycling Plan", specifying the manner in which each ship will be recycled, depending on its particulars and its inventory. Parties will be required to take effective measures to ensure that ship recycling facilities under their jurisdiction comply with the Convention, (**IMO, 2019**). **Brennecke *et al.* (2016**) commented that microplastics can play an important vector role in heavy metal transport and considered copper (Cu) and zinc (Zn) transport from antifouling system. Interestingly, **Auta,Emenike and Fauziah (2017)** also reviewed microplastics as a pollution pathway and commented that metals from anti-fouling compounds bonded to plastics, though neither study considered plastics from anti-fouling systems or other marine coatings themselves. Hull cleaning activities are generally associated with large ports and, potentially in a more unregulated fashion, recreational marinas. There is concern and impact associated with pollution from antifouling system and research has shown that copper release increases significantly with underwater cleaning of antifouling systems (**Schiff, Diehl and Valkirs, 2004; Earley *et al.*, 2014)**.

Some metals are essential for normal cellular growth (nickel, zinc and copper), whereas others don't have any known cellular role for the moment (**Kiefer, 2000**).Sea organisms that take up dissolved and particulate metals may be used as indicators of the bioavailability, over time, of a specific pollutant (**Volterra and Conti, 2000**). This information is very difficult to obtain by analyses of water and sediments. Bio-indicators respond especially to those fractions that have an obvious ecotoxicological relevance, particularly heavy metals (**Rainbow and Phillips, 1993; Conti, 2002**). Accumulative indicators (or bio-accumulators) have the property of accumulating contaminants in the tissues and are used as an integrated measure of their concentration in the environment. They have to be widely distributed geographically, be available all year, be very tolerant to the type of contamination (they must not be killed by the contaminant itself), be easy to sample and preserve and, above all, show a positive correlation between the concentration of contaminant accumulated in their organism and the concentration of the contaminant in the surrounding environment (**Phillips, 1977; Conti and Cecchetti, 2001**). The biomarker concept has been associated with an 'early warning' of pollution-induced stress. This early warning may have two meanings. First, it may indicate the detection of pollutant effects early in time, e.g. shortly after emission has started, the warning being that effects may develop with time at higher levels of biological organization (e.g. populations and ecosystems). Second, it may indicate the

detection of a response at concentrations below those causing irreversible effects. biomarkers can be used in the assessment of environmental "damage" and in the formulation of regulations to control such damage'. In our view, biomarkers can tell us that there is a deviation from health at the individual level, but whether this will result in effects at higher levels of biological organization, remains to be seen, **(Marcelo and Marta, 2007)**. Although dead algae have been utilized successfully in heavy metal adsorption experiments (**Leusch *et al*., 1995** and **Holan *et al*., 1998**), living algae remove significantly more metal ions than non-living algae at all metal concentrations examined, probably due to metabolic uptake and continuous growth (**Maeda and Skaguchi, 1990** and **Terry and Stone, 2002**). Moreover, living algae possess intracellular polyphosphates which participate in metal sequestration, as well as algal extracellular polysaccharides that serve to chelate or bind metal ions (**Van Eykelenburg, 1978; Kaplan *et al*., 1987; Zhang and Majidi, 1994** and **Gardea-Torresdey *et al*., 1996a**).

Biological monitoring or biomonitoring can be defined as the systematic use of biological responses to evaluate changes in the environment, with the intent of establishing a quality control program (**Cairns and van der Schalie, 1980**). Marine microalgae are particularly promising indicator species for organic and inorganic pollutants since they are typically the most abundant life forms in aquatic environments and occupy the base of the food chain. The challenge, however, is that the low levels of pollutants regularly present in individual cells may not be sufficient to induce significant biochemical adaptations in microalgae, whereas biomagnification/bioaccumulation through the food web may cause drastic impacts on organisms at higher levels (**Moacir *et al*., 2008**). Using of marine photosynthetic organisms like algae have increasingly been used as biodetectors to different marine pollutants (**Conti** and **Cecchetti**; **Conti *et al*., 2007**). Because of its natural and widespread occurrence along worldwide seashores, photosynthesizing organisms could be useful for a time-integrated picture of the ecosystem response to exposure to toxic compounds. Both, macroalgae (**Fytianos *et al*., 1999 and Conti and Cecchetti, 2003)** and microalgae (**Mallick, 2004 and Tripathi *et al*., 2006**) are important tools to monitor physiological changes in the presence of heavy metals. Algae are able to absorb pollutants from the aquatic environment and biotransform organic compounds and immobilize inorganic elements to make them less toxic (**Sa´nchez-Rodrı´guez *et al*., 2001**).

As a result of their substantial biomass and comparatively large surface-to-volume ratio, microalgae play a major role in the biogeochemical cycling of nutrients and pollutants in the oceans (**Van Gestel and Van Brummelen, 1996**; **Okamoto and Colepicolo, 1998)**. They have been referred to as a ‘‘green liver’’ of the oceans, acting as important sinks for environmental chemical compounds (**Sandermann, 1992**).

Biomarkers are defined as quantitative measures of changes in the biological system that can be related to exposure to the toxic effects of environmental chemicals (**WHO, 1993; Peakall and Walker, 1994**). Bioindicator: an organism giving information on the environmental conditions of its habitat by its presence or absence and its behaviour. (**IMO/ FAO/ Unesco/ WMO/ WHO/ IAEA/UN/UNEP, 1986**). It has been confirmed that inhibition of growth and photosynthesis are

the basic reflex of the toxic effects of pollutants on microalgae (**Franqueira *et al*., 2000**). Moreover, toxicity tests based on algae have been used in conjunction with other organisms to assess associated environmental effects of pollutants and the integrity of aquatic ecosystems (**Cid *et al*., 1996; Blaise and Menard, 1998**).

The aim of this work is to estimate the stimulatory or inhibitory effects of three heavy metals namely nickel, zinc and copper on the growth of the marine unicellular green alga *Dunaliella tertiolecta*. The work was designed to assess the effect of different concentrations of the three pollutants on growth of alga depending on cell number and growth rate.

Materials and methods

A-Biological material:

The biological material used in this thesis was the unicellular marine green alga, *Dunaliella tertiolecta*, which was obtained from UTEX-the culture collection of algae, at the University of Texas at Austin, USA.

a- Culture conditions:

Each of the axenic cultures was grown in 50 ml MH medium in 250 ml Erlenmeyer Pyrex-glass flasks under controlled laboratory conditions (temperature at 25˚C ± 3˚C and light at 4000 lux) in a controlled culturing chamber. Each experimental culture flask was regularly swirled daily by hand to detach adhered algal cells from the walls of the flasks.

b- Culture medium:

The axenic cultures of *Dunaliella tertiolecta* was grown on MH medium (**Loeblich, 1982**) as cleared in table (A).

Table (A): Amount of salts per liter of MH medium.

Salts	Amount per liter
NaCl	73.050 g
$MgCl_2.6H_2O$	1.500 g
$MgSO_4.7H_2O$	0.500 g
KCl	0.200 g
$CaCl_2.2H_2O$	0.200 g
KNO_3	1.000 g
$NaHCO_3$	0.043 g
$*KH_2PO_4$	0.035 g
EDTA	1.890 mg
$FeCl_3.6H_2O$	2.440 mg
$ZnCl_2$	0.041 mg
H_3BO_3	0.610 mg
$CoCl_2.2H_2O$	0.015 mg
$CuCl_2.2H_2O$	0.041 mg
$MnCl_2.4H_2O$	0.410 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.300 mg

pH was adjusted at 7.5
*potassium phosphate solution was autoclaved separately and added aseptically to the sterilized medium to avoid phosphate precipitation.

c- Harvesting of culture:

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, pigment fractions, carbohydrates, glycerol, protein, protein profile, isozymes, fatty acids and amino acids contents.

d- Determination of growth parameters:

***Cell counts:**

Growth determination by cell count of the tested organism was determined by using the hemacytometer slide. The later consists of 16 small squared areas, each of 0.0025 mm^2 and 0.1mm depth. The total volume of the big square was 0.004 mm^3. By means of a sterilized pipette a drop of homogenous algal suspension was transferred to the hemacytometer slide, the cover was put and the cells were counted microscopically after setting of cells (after 2 minutes). At least 10 replicates were taken to get the mean number of cells per ml culture.

***Growth rate:**

The growth rate (number of divisions/day) was calculated by using the formula proposed by **Robert (1979)**:

$$R = \left(\frac{3.322}{t_2 - t_1}\right) \cdot \left(\log \frac{N_2}{N_1}\right)$$

Where: 3.322 = growth constant.

t_1 = time at the beginning of the first measurement. t_2 = time of the next measurement.

N_1 = number of cells/ml culture at t_1. N_2 = number of cells/ml culture at t_2.

B- <u>Heavy metals bioassay</u>:

Three heavy metals including copper, zinc, and nickel were selected during the present study based on their abundance in the industrial waste water around Alexandria, as well as, their effect on the receiving aquatic ecosystems. Stock solutions of the selected heavy metals were prepared from their salts in double distilled water and sterilized by filtration through 0.2 µm nitrocellulose membranes. Table (B) illustrates weights of stock metals salt dissolved in one liter of double distilled water and the different concentrations of the selective heavy metals used in the metal bioassays.

According to **Wong and Pak (1992)**, a preliminary experiment using a wide range of metal solutions [$CuSO_4.5H_2O$, $ZnCl_2$ and $NiCl_2$] were carried out to determine the suitable concentrations of these 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.

Table (B):Weights of stock metals salt (g/L) and the different concentrations of the three heavy metal ions tested (Ni^{2+}, $Zn^{2+}$and Cu^{2+}).

Metal salts	Weights of metal salts g/L	Concentrations used (mg/L)				
$CuSO_4.5H_2O$	3.9294	5	10	15	20	25
$ZnCl_2$ (anhydrous)	2.0846	5	10	15	20	25
$NiCl_26H_2O$	4.0501	5	10	15	20	25

Results & discussion:

Growth parameters of *Dunaliella tertiolecta* measured by cell number and growth rate cultured for 16 days:-

The results recorded in table (1) and figure (1) showed that growth parameters calculated from the number of cells cleared that growth rate increased gradually till the 8th day of culturing where it reached maximum value then it began to decrease reaching minimum value at the 16th day. Growth rate was 0.473 at the 8th day while it dropped to 0.013 at the 16th day. Values of growth parameters measured as cell number and growth rate which have been used as indices for growth of *Dunaliella tertiolecta* cultured in the basal medium showed linear relationship. This coincides with the results obtained by **Ben-Amotz and Avron (1981); El-Maghrabi, (1997)** and **Sahar, (1998)**.

Table (1):Growth parameters based on cell number and growth rate of *Dunaliella tertiolecta* cells cultured for 16 days.

Time (days)	Cells No.X10^6 (cell/ml)	Growth Rate
0	0.220	–
2	0.250	0.092
4	0.360	0.263
6	0.540	0.292
8	1.040	0.473
10	1.400	0.214
12	2.190	0.323
14	2.860	0.193
16	2.910	0.013

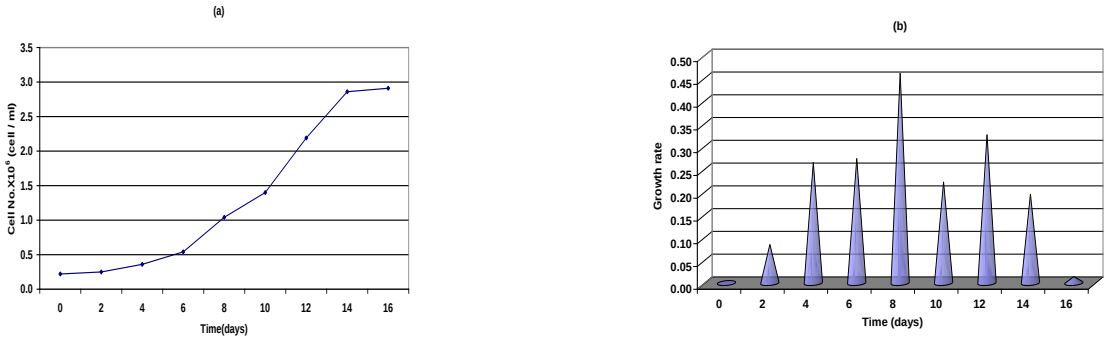

Figure (1):Growth parameters measured (a) as cell number (b) as growth rate of *Dunaliella tertiolecta*cells cultured for 16 days on basal medium.

Effect of different concentrations of nickel, zinc and copper on growth parameters of *Dunaliella tertiolecta* measured by cell number and growth rate cultured for 16 days:-

A- Nickel:

Ni^{2+} ions are known to be essential cofactors for bacterial and algal enzymes (**Hausinger, 1987** : **Luis, 1984** and **Thauer, 1983**). The results recorded in table(2-A&B) and figure (2) concerning growth parameters cleared that at the 8th day of culturing and at 5 mg/L $Ni^{2+}$the number of cells increased by 46.2% compared to control. At the 12th day of culturing the number of cells increased by 9.6% compared to control. At the end of the experiment (the 16th day) the number of cells at 5mg/L Ni^{2+} decreased by 3.1% compared to control.

Table (2-A): Number of cells ($X10^6$) of *Dunaliella tertiolecta* cultured for 16 days at different concentrations of Ni^{2+} (mg/L).

Time (days)	Control	Different Concentrations of Ni^{2+} (mg/L)					F (p)	LSD
		5	10	15	20	25		
0	0.220±0.002 [a]	0.220±0.002 [a]	0.220±0.002 [a]	0.220±0.002 [a]	0.220±0.002 [a]	0.220±0.002 [a]	0.000 (1.000)	0.002
2	0.250±0.001 [a]	0.270±0.004 [b]	0.270±0.001 [b]	0.260±0.004 [c]	0.260±0.003 [d]	0.240±0.001 [e]	52.340** (<0.001)	0.003
4	0.360±0.003 [a]	0.360±0.003 [a]	0.350±0.003 [b]	0.340±0.001 [c]	0.270±0.002 [d]	0.260±0.003 [e]	1472.308** (<0.001)	0.003
6	0.540±0.001 [a]	0.630±0.001 [b]	0.580±0.003 [c]	0.510±0.003 [b]	0.500±0.001 [d]	0.430±0.002 [e]	5103.000** (<0.001)	0.002
8	1.040±0.003 [a]	1.520±0.003 [b]	1.300±0.001 [c]	0.870±0.002 [d]	0.870±0.005 [d]	0.600±0.003 [e]	3530.429** (<0.001)	0.003
10	1.400±0.002 [a]	1.680±0.002 [b]	1.490±0.003 [c]	1.380±0.002 [d]	0.890±0.003 [e]	0.620±0.002 [f]	147406.88** (<0.001)	0.003
12	2.190±0.003 [a]	2.400±0.001 [b]	2.190±0.002 [a]	1.720±0.004 [c]	1.400±0.001 [d]	0.500±0.004 [e]	204049.09** (<0.001)	0.003
14	2.860±0.001 [a]	2.900±0.004 [b]	2.840±0.003 [c]	2.010±0.003 [d]	1.300±0.003 [e]	0.500±0.001 [f]	41.5865.58** (<0.001)	0.003
16	2.910±0.004 [a]	2.820±0.002 [b]	2.810±0.003 [c]	2.020±0.002 [d]	1.200±0.003 [e]	0.400±0.003 [f]	456256.74** (<0.001)	0.003

F (p) : F-test (ANOVA) and its significance between groups
* : Statistically significant at $p \leq 0.05$
Different superscripts are significant

LSD : Least significance difference at 0.05
** : Statistically significant at $p \leq 0.01$
Data are expressed in Mean ± SD

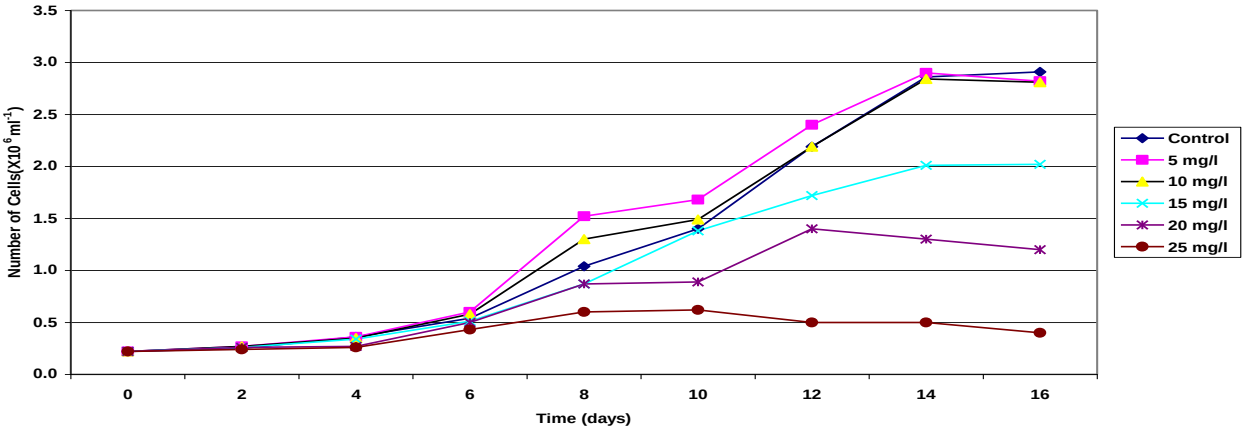

Figure (2): Effect of different concentration of Ni^{2+} (mg/L) on number of cells ($\times 10^6$) of *Dunaliella tertiolecta* cultured for 16 days.

This data indicates that low concentrations of Ni^{2+} ions (5 & 10 mg/L) stimulate the growth of *Dunaliella tertiolecta* from the beginning to the 14th day of culturing compared to control, while at the higher concentrations (15, 20 & 25 mg/L) was inhibited from the beginning to the end of the experiment.

Table (2-B):Growth rate of *Dunaliella tertiolecta* cultured for 16 days at different concentrations of Ni^{2+} (mg/L)

Day No	Control	5 mg/L	10 mg/L	15 mg/L	20 mg/L	25 mg/L
0	---	---	---	---	---	---
2	0.092	0.148	0.148	0.121	0.121	0.063
4	0.263	0.208	0.187	0.194	0.027	0.058
6	0.292	0.368	0.364	0.292	0.268	0.363
8	0.473	0.671	0.582	0.675	0.400	0.240
10	0.214	0.072	0.098	0.333	0.016	0.024
12	0.323	0.257	0.278	0.159	0.327	–
14	0.193	1.365	0.187	0.112	–	0.000
16	0.013	–	–	0.004	–	–

At 20 mg/L of $NiCl_2$, no significant effect on either lag phase or culture doubling of the blue-green alga *Nostoc linckia* was found, but at 10 mg/L the growth was totally inhibited (**kumar *et al.*, 1985**). Similar results were also obtained by **Stratton and Corke (1979)** who reported that high concentrations of Ni^{2+} were also toxic to *Chlorella* and *Anabaena* species. Also, **El-Mazally (2002)** found that low concentrations of Ni^{2+} stimulated the growth of *Scenedesmus obliques* compared to control. Progressive increase in the concentration of Ni^{2+} inhibited the

different measured growth parameters. Our results go coincident with those obtained by **Jelan (2017)**, she found that up take of nickel by *Dunaliella salina* in industrial effluent containing 12.4 mg/L Ni^{+2} reached 57% in 180 minutes of treatment.

B- Zinc:

Anent effect of different concentrations of Zn^{2+} ions on growth parameters of *Dunaliella tertiolecta* the results recorded in tables (3A) & 3B and figure (3) revealed that Zn^{2+} ions are more toxic than Ni^{2+} ions. At the 8^{th} day of culturing the growth of the organism in case of 5 mg/L Ni^{2+} increased by 46.2% while for Zn^{2+} at the same concentration the number of cells increased by 34.7% compared to control, even at the 12^{th} day of culturing and at the same concentration of both Ni^{2+} and Zn^{2+} the number of cells increased by 9.6% and 7.3% respectively. Also at the end of the experiment the growth of the organism in case of 5 mg/L Ni^{2+} decreased only by 3.1%, while for Zn^{2+} it decreased by 3.8%. At 10 mg/L Zn^{2+} the number of cells increased gradually till the 12^{th} day of culturing compared to control then it began to decrease. The only difference between the increase in number of cells at control , 5 and 10 mg/L Zn^{2+} is that the cell number obtained at the 14^{th} to the 16^{th} day in case of control increased, while in case of the other two concentrations (5&10 mg/L) of Zn^{2+} ,the number of cells decreased compared to control. On the contrary in case of 15, 20, 25 mg/L Zn^{2+} the number of cells decreased compared to control from the beginning to the end of the experiment (the 16^{th} day) but the rate of decrease was more prominent at 25 mg/L than at 15 and 20 mg/L Zn^{2+}.

At lower concentrations (5 and 10 mg/L) the rate of growth increased by 40.83% and 7.3% respectively compared to control. The only difference in the rate of increase between control, 5 and 10 mg/L Zn^{2+} is that at the 14^{th} to 16^{th} days in case of control it increased while at 5 and 10 mg/L Zn^{2+} the rate of growth decreased. On the contrary, at 15, 20 and 25 mg/L Zn^{2+} the rate of growth decreased from the beginning to the end of the experiment but the rate of decrease was more prominent at 25 mg/L than at 15 and 20 mg/L Zn^{2+}. It is clear also that number of cells of *Dunaliella tertiolecta* in the presence of 5 mg/L Zn^{2+} was higher than control itself and nearly similar to control at 10 mg/L. These results nearly coincide with those obtained by many authors, **Jelan, (2017),** reported that *Dunaliella salina* was effective alga in bioremoval of *Zinc* from both petrochemicals (87.4 % after 180 min.) and fertilizer (80.9 % after 180 min.) effluent that contained 14.04 and 10.41 mg/L of Zn^{+2} respectively, and algal growth did not affected by these concentrations. **Fisher and Jones, (1981)** reported an increase in growth of *Asteriorella japonica* in response to elevated levels of copper and zinc. **El-Naggar (1993)** indicated that higher concentrations of zinc reduced the growth of *Chlorella vulgaris* and *Scenedesmus bijuga*.

Table (3-A): Number of cells (X10^6) of *Dunaliella tertiolecta* cultured for 16 days at different concentrations of Zn^{2+} (mg/L).

Time (days)	Control	Different Concentrations of Zn^{2+} (mg/L)					F (p)	LSD
		5	10	15	20	25		
0	0.220±0.002 ^{a}	0.220±0.001 ^{a}	0.220±0.002 ^{a}	0.220±0.002 ^{a}	0.220±0.002 ^{a}	0.220±0.002 ^{a}	0.000 (1.000)	0.003
2	0.250±0.001 ^{a}	0.250±0.003 ^{a}	0.250±0.002 ^{a}	0.250±0.004 ^{a}	0.240±0.001 ^{b}	0.230±0.002 ^{c}	37.059** (<0.001)	0.003
4	0.360±0.003 ^{a}	0.360±0.001 ^{a}	0.370±0.004 ^{b}	0.320±0.001 ^{c}	0.350±0.003 ^{d}	0.450±0.003 ^{e}	812.857** (<0.001)	0.004
6	0.540±0.001 ^{a}	0.561±0.003 ^{b}	0.544±0.001 ^{c}	0.426±0.003 ^{a}	0.505±0.002 ^{d}	0.480±0.001 ^{e}	15207.000** (<0.001)	0.003
8	1.040±0.003 ^{a}	1.401±0.004 ^{b}	1.040±0.002 ^{a}	1.020±0.001 ^{c}	0.750±0.003 ^{d}	0.500±0.004 ^{e}	31197.000** (<0.001)	0.005
10	1.400±0.002 ^{a}	1.550±0.002 ^{b}	1.400±0.004 ^{a}	1.220±0.004 ^{c}	0.780±0.001 ^{d}	0.520±0.003 ^{e}	57015.000** (<0.001)	0.004
12	2.190±0.003 ^{a}	2.350±0.002 ^{b}	2.200±0.003 ^{c}	2.180±0.002 ^{d}	1.020±0.003 ^{e}	0.540±0.004 ^{f}	225033.191** (<0.001)	0.004
14	2.860±0.001 ^{a}	2.800±0.004 ^{b}	2.250±0.001 ^{c}	2.180±0.003 ^{d}	1.020±0.002 ^{e}	0.620±0.003 ^{f}	370898.571** (<0.001)	0.004
16	2.910±0.004 ^{a}	2.800±0.001 ^{b}	2.300±0.004 ^{c}	1.850±0.003 ^{d}	0.980±0.004 ^{e}	0.660±0.003 ^{f}	262496.000** (<0.001)	0.005

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

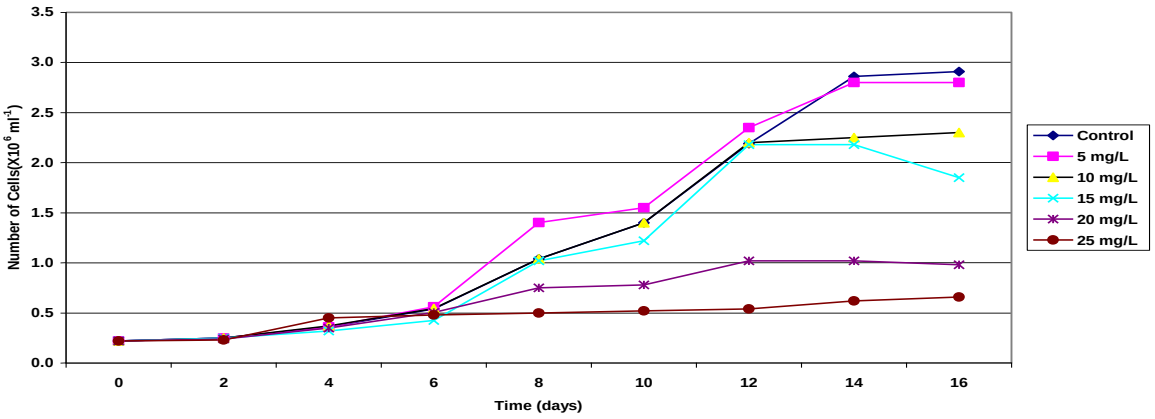

Figure (3): Effect of different concentration of Zn^{2+} (mg/L) on number of cells (×10^6) of *Dunaliella tertiolecta* cultured for 16 days.

Table (3-B): Growth rate of *Dunaliella tertiolecta* cultured for 16 days at different concentrations of Zn^{2+} (mg/L).

Day No	Control	5 mg/L	10 mg/L	15 mg/L	20 mg/L	25 mg/L
0	---	---	---	---	---	---
2	0.092	0.092	0.092	0.092	0.063	0.032
4	0.263	0.263	0.283	0.178	0.272	0.484
6	0.292	0.320	0.653	0.207	0.264	0.047
8	0.473	0.660	0.467	0.630	0.286	0.029
10	0.214	0.073	0.214	0.129	0.028	0.028
12	0.323	0.300	0.326	0.419	0.194	0.027
14	0.193	0.126	0.016	–	–	0.100
16	0.013	–	0.016	–	–	0.045

C- Copper:

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, Fitzgerald 1974, Whittaker *et al*., 1978, Stockes, 1983; Sorentino, 1979; Stauber and Florence, 1985a; Wong and Chang, 1991; Abdel-Hamid *et al*., 1992; Vymazal, 1995**). It is clear from the results obtained in table (4A &4B) and figure (4) that low concentration of Cu^{2+} ions (5mg/L) stimulated the growth of this organism compared to control. At the 8^{th} day of culturing and under the effect of 5mg/L Cu^{2+} the organism reached its maximum rate of growth (0.607) i.e. increased by 28.3% compared to control. On the contrary, at concentration 10 mg/L Cu^{2+} a slight decrease were recorded in the measured growth parameters. It is worth mentioning here that at 10 mg/L Cu^{2+} the number of cells increased gradually but slightly from the beginning of the experiment to the 8^{th} day. This slight increase at the 8^{th} day of culturing was 7.7% compared to control while at 5 mg/L Cu^{2+} the number of cells increased by 27.1% compared to control. However, by increasing the period of culturing from the 8^{th} day to the 16^{th} day, the number of cells decreased gradually reaching the minimum values at the end of the experiment compared to control. Also, **Stauber and Florence (1985a)** reported that copper at concentrations higher than 5 mg/L inhibited the growth of *Nostoc closterium* by 50% below control. The number of cells at the end of the experiment at 10 mg/L Cu^{2+} decreased by 31.6% compared to control and 28.9% compared to 5 mg/L. Copper was found to be more toxic to the dinoflaellate *Prorocentrum micans* than to the diatom, *Nitzschia closterium* as reported by **Carpene and Boni (1992).** 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)**. Further increase in concentration of Cu^{2+} i.e. at 15, 20, 25 mg/L Cu^{2+} all parameters of growth decreased gradually from the first two days till the end of the experiment.

This decrease in growth parameters was more prominent at 20 and 25 mg/L Cu^{2+}. Even at the last four days of culturing (from the12th to the16th day) the organism disappeared completely at concentration 25 mg/L. The percentage of decrease at the 8th day reached 51.9% and 66.3% at concentrations 20 and 25 mg/L respectively compared to control. Also, **Jelan (2017)**, reported significant decrease in Cu^{2+} up take by *Dunaliella salina* in two industrial effluents contain 18.48 and 12.31 mg/L of Cu^{2+} respectively, this result indicate that Cu^{2+} is more toxic to *Dunaliella salina* than Ni^{2+} and Zn^{2+}.

Table (4-A): Number of cells (X10^6) of *Dunaliella tertiolecta* cultured for 16 days at different concentrations of Cu^{2+} (mg/L).

Time (days)	Control	Different Concentrations of Cu^{2+} (mg/L)					F (p)	LSD
		5	10	15	20	25		
0	0.220±0.002 ^{a}	0.220±0.002 ^{a}	0.220±0.002 ^{a}	0.220±0.002 ^{a}	0.220±0.002 ^{a}	0.220±0.002 ^{a}	0.000 (1.000)	0.002
2	0.250±0.001 ^{a}	0.250±0.003 ^{a}	0.250±0.002 ^{a}	0.250±0.003 ^{a}	0.240±0.002 ^{b}	0.230±0.003 ^{c}	37.059** (<0.001)	0.003
4	0.360±0.003 ^{a}	0.380±0.001 ^{b}	0.320±0.003 ^{c}	0.320±0.001 ^{c}	0.280±0.002 ^{d}	0.310±0.001 ^{e}	1167.000** (<0.001)	0.002
6	0.540±0.001 ^{a}	0.570±0.003 ^{b}	0.550±0.002 ^{c}	0.520±0.003 ^{d}	0.480±0.004 ^{e}	0.400±0.003 ^{f}	1570.909** (<0.001)	0.003
8	1.040±0.003 ^{a}	1.322±0.002 ^{b}	1.120±0.001 ^{c}	0.720±0.004 ^{a}	0.500±0.003 ^{d}	0.350±0.004 ^{e}	50750** (<0.001)	0.004
10	1.400±0.002 ^{a}	1.500±0.001 ^{b}	1.400±0.004 ^{a}	1.220±0.002 ^{c}	0.480±0.002 ^{d}	0.300±0.054 ^{e}	1534.130** (<0.001)	0.025
12	2.190±0.003 ^{a}	2.200±0.003 ^{b}	2.050±0.002 ^{c}	1.180±0.001 ^{d}	0.420±0.004 ^{e}	0.300±0.001 ^{f}	391050.81** (<0.001)	0.003
14	2.860±0.001 ^{a}	2.760±0.003 ^{b}	2.050±0.003 ^{c}	1.020±0.004 ^{d}	0.370±0.001 ^{e}	-	441736.76** (<0.001)	0.003
16	2.910±0.004 ^{a}	2.790±0.004 ^{b}	1.990±0.001 ^{c}	1.010±0.003 ^{d}	0.370±0.003 ^{e}	-	334123.40** (<0.001)	0.003

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

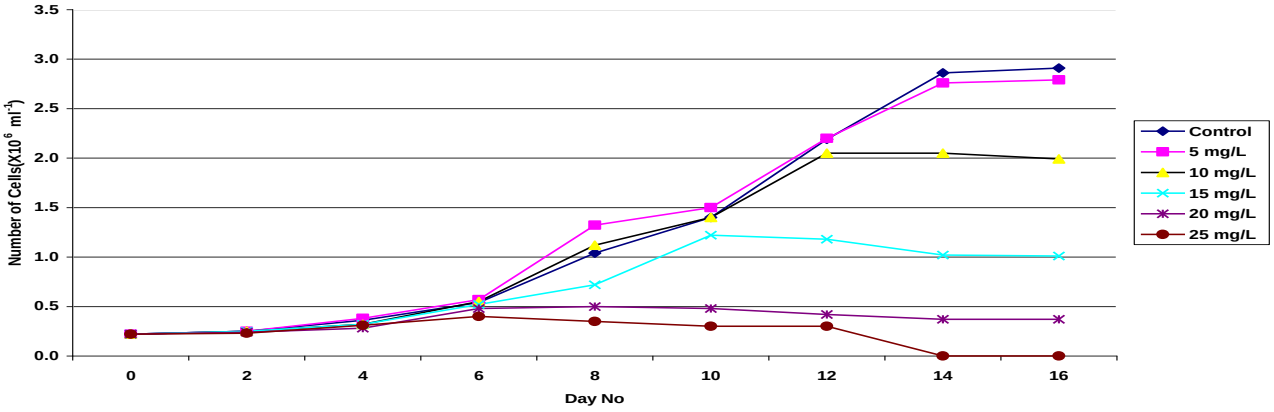

Figure (4): Effect of different concentration of Cu^{2+} (mg/L) on number of cells (×10^6) of *Dunaliella tertiolecta* cultured for 16 days.

Table (4-B): Growth rate of *Dunaliella tertiolecta* cultured for 16 days at different concentrations of Cu^{2+} (mg/L).

Day No	Control	5 mg/L	10 mg/L	15 mg/L	20 mg/L	25 mg/L
0	---	---	---	---	---	---
2	0.092	0.092	0.090	0.092	0.063	0.032
4	0.263	0.300	0.178	0.178	0.111	0.215
6	0.292	0.290	0.390	0.350	0.389	0.184
8	0.473	0.607	0.513	0.235	0.029	–
10	0.214	0.091	0.161	0.380	–	–
12	0.323	0.276	0.275	–	–	–
14	0.193	0.160	–	–	–	–
16	0.013	0.010	–	–	–	–

At the cell membrane, copper may interfere with cell permeability or the binding of essential metals **(Huntsman and Sunda, 1980 and Suwalsky *et al*., 1998).** Following copper transport into the cell, copper may react with SH enzyme groups disrupting enzyme active sites and cell division **(Fisher and Jones, 1981; Florence and Stauber, 1986 and Nies, 1999).** The difference in metal toxicity could be attributed to differential affinities of the cations for sulfur complexation. Metals with high affinity for sulfur such as mercury and copper are expected to be more toxic than metals exhibited lower affinity for sulfur such as cadmium **(Nies, 1999)**. Concomitantly, copper produces irreversible damage to chloroplast lamellae preventing photosynthesis, inhibiting carbohydrates, cell division and eventually causing death **(Maloney and Palmer, 1956, Sunda and Guillard, 1976 and Chiaudani and Vighi, 1978).** However, further increase of Cu^{2+} concentration led to gradual inhibition in all the previous metabolic processes for the studied organism. **Huda *et al.*, 2014** reported Zinc (Zn) was found the most abundant element in wastewater (179.12 mg/L), while Ni was less abundant (19.14 mg/L) and copper Cu recorded relatively high concentrations 62.17 mg/L, and the heavy metal uptake by *Dunaliella* alga differed according to the metal type, the up take in case of Zn and Ni reached nearly 94% for both heavy metals and 90% in case of Cu.

Summary

Environmental pollution with heavy and toxic metals, produced by mining, processes of metallurgy and other chemical industries is a worldwide problem to human health and environment because of their high concentrations in the biological food chains and long term presence in the ecosystems. Biomarkers are used to measures of changes in the biological system that can be related to exposure to the toxic effects of environmental chemicals. The biomarker concept has been associated with an 'early warning' of pollution-induced stress. This early warning may have two meanings. First, it may indicate the detection of pollutant effects early in time. Second, it may indicate the detection of a response at concentrations below those causing irreversible effects. Biomarkers can be used in the assessment of environmental "damage" and in the formulation of regulations to control such damage'. Bioindicator: an organism giving information on the environmental conditions of its habitat by its presence or absence and its behavior. Moreover, toxicity tests based on algae have been used in conjunction with other organisms to assess associated environmental effects of pollutants and the integrity of aquatic ecosystems It has been confirmed that inhibition of growth and photosynthesis are the basic reflex of the toxic effects of pollutants on microalgae. Because *Dunaliella* sp is a single-celled, photosynthetic green alga, that is characteristic for its ability to outcompete other organisms and thrive in hypersaline environments, the work designed to estimate the stimulatory or inhibitory effects of three heavy metals (nickel, zinc and copper) on the growth of the marine unicellular green alga *Dunaliella tertiolecta* which used as biological biomarker for different pollutants. Three heavy metals were selected during the present study based on their abundance in the industrial waste water around Alexandria, as well as, their effect on the receiving aquatic ecosystems, also they considered essential micro-element for growth of different algal species. The results proved that Ni^{2+} ions were less toxic than both Zn^{2+} and Cu^{2+} ions on the growth of *Dunaliella tertiolecta* which used as biological biomarker for different pollutants. This idea could be confirmed from the results obtained concerning growth parameters. After 8 days of culturing, the obtained growth parameters in case of 5mg/L Ni^{2+} increased by 46.2% while in case of Zn^{2+} and Cu^{2+} they increased by 34.7% and 27.1% respectively compared to control. The results obtained at the 12^{th} day of culturing supported also this idea. At the end of the experiment, the growth parameter (number of cells) in case of Ni^{2+} decreased by 3.1% while at Zn^{2+} the same parameter of growth decreased by 3.8%. From these entire results it could be concluded that Ni^{2+} ions are less toxic than Zn^{2+} ones and the later is less toxic than Cu^{2+} ions on the growth of *Dunaliella tertiolecta*.

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