

**CADMIUM INDUCED OXIDATIVE STRESS IN *DUNALIELLA SALINA***A. Moradshahi<sup>\*1</sup> and E. Yazdanpanah<sup>2</sup><sup>1</sup>Department of Biology, Faculty of Sciences, Shiraz University, 71454 Shiraz, Islamic Republic of Iran,<sup>2</sup>Department of Biology, Payame Noor University, Tehran, Iran

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**ABSTRACT**

The unicellular green algae *Dunaliella salina* contains various antioxidants which protect the cell from oxidative damage due to environmental stresses such as heavy metal stress. In the present study, the response of *D. salina* at the stationary growth phase to oxidative stress generated by cadmium chloride was investigated. Growth, expressed as cell number per ml of culture, did not change up to 75  $\mu\text{M}$  cadmium but decreased significantly at 100 and 150  $\mu\text{M}$   $\text{Cd}^{2+}$ . Reduction in chlorophyll content and carotenoids content per ml of culture was observed in the presence of  $\text{Cd}^{2+}$ . Total antioxidant activity, expressed as  $\mu\text{mole}$  Trolox equivalent per  $10^6$  cell ( $\mu\text{mol TE } 10^6 \text{ cell}^{-1}$ ) and also total phenolic content ( $\text{pg GAE, cell}^{-1}$ ) were significantly reduced in the presence of  $\text{Cd}^{2+}$ . Lower  $\text{Cd}^{2+}$  concentration had no influence on the activity of ascorbate peroxidase, 100 and 150  $\mu\text{M}$   $\text{Cd}^{2+}$  caused significant reduction in enzyme activity. Lipid peroxidation, reported as malondialdehyde content, was the same as control up to 100  $\mu\text{M}$   $\text{Cd}^{2+}$  but increased at higher  $\text{Cd}^{2+}$  concentration. It is concluded that high concentration of cadmium have negative effect on aquatic algae.

**Keywords:** *Dunaliella salina*, Carotenoids, Antioxidant activity, Cadmium chloride, Lipid peroxidation, Ascorbate peroxidase

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## 1. INTRODUCTION

One of the major environmental problems facing the industrial world is contamination of air, soil and water with heavy metals (Nriagu 1990). Heavy metals have profound negative effects on plant and micro-organisms growth (Yadav 2010). The primary response of plants and algae to heavy metal toxicity is the production and accumulation of reactive oxygen species (ROS). ROS are responsible for the damage to membrane and other essential biomolecules such as proteins, DNA and lipids or inhibit the activity of several enzymes (Mithofer et al. 2004; Hall 2000).

Cadmium (Cd) is a non-essential element, which seems to be more or less toxic to plants and micro-organisms (Benavides et al. 2005; Gratao et al. 2005). Cd can produce ROS directly via the Fenton and Haber-Weiss reactions and indirectly by inhibiting antioxidant enzymes (Romero-Puertas et al. 2007).

Studies performed in different plant species have showed that Cd can interfere with a number of metabolic processes such as photosynthesis, respiration or nutritional status (Poschenrieder and Barcelo 1999; Sandalio et al. 2001). Although the mechanisms involved in its toxicity has not been well established (Romero-Puertas et al. 2007). Cadmium can induce oxidative stress by the generation of ROS, such as  $H_2O_2$ ,  $O_2^{\cdot-}$  radicals, and  $OH^{\cdot}$  radical, as well as disturbances in the antioxidative systems for the detoxification in ROS (Mithofer et al. 2004; Rodriguez-Serrano et al. 2006; Schützendübel et al. 2001). Cd can inactivate enzymes reacting with Cys residues (Schützendübel and Polle, 2002).

To reduce the effects of oxidative stress, organisms have evolved a complex antioxidant system, which is composed of low molecular weight antioxidant such as ascorbate, glutathione and carotenoids, as well as ROS-scavenging enzymes such as catalase, ascorbate peroxidase and superoxide dismutase (Abd El-Baky et al. 2002; Abd El-Baky et al. 2003; Abd El-Baky et al. 2004).

The unicellular green algae *Dunaliella* is widely distributed throughout the world and for the first time Ginzburg and Ginzburg reported its presence in Maharlu salt lake in Shiraz, Iran (1984). Since the effects of heavy metals on ROS metabolism in alga are different (Pinto et al. 2003) and information on the response of *Dunaliella* to Cd is rare, to evaluate the sensitivity of *Dunaliella salina* to Cd, in this work the effect of  $CdCl_2$  on the growth, pigmentation, lipid peroxidation, ascorbate peroxidase activity, antioxidant potential and total phenolic compound are studied.

## 2. MATERIALS AND METHODS

### 2.1. Algal isolation and CdCl<sub>2</sub> treatments

*D. salina* was isolated from water samples collected from Maharlu salt lake 28 Km southeast of Shiraz, Iran. Algae was purified in sterilized nutrient media as described by Ben-Amotz and Avron (1990). Cells were treated with 25, 50, 75, 100 and 150 µM CdCl<sub>2</sub> at late logarithmic growth phase. The cultures were kept under continuous light with an intensity of 3000 ft-candle at 22°C. After 24, 48, and 72 hours, samples were taken for growth measurements. Total chlorophyll and carotenoids, lipid peroxidation, antioxidant capacity and ascorbate peroxidase activity were determined 48 hours after exposure to cadmium chloride.

### 2.2. Growth measurements

Cell counts were carried out using a haemocytometer under a light microscope (Nikon Eclipse 8i) and reported as the number of cells mL<sup>-1</sup> of the growth culture.

### 2.3. Total chlorophylls and carotenoids determination

3 mL of algal suspension was centrifuged at 5000 g for 10 min and pigments were extracted from the pellet by 80% acetone. After centrifugation total chlorophylls and carotenoids were determined based on the method of Lichtenthaler and Buschmann (2001).

### 2.4. Total antioxidant activity

Antioxidant activity measurements were done as described by Thaipong et al. (2006). In brief, the FRAP reagent contained 2.5 mL of 10 mM TPTZ (2, 4, 6-tripyridyl-S-triazine) solution in 40 mM HCl, 2.5 mL of 20 mM FeCl<sub>3</sub> and 25 mL of 300 mM acetate buffer pH 3.6. It was freshly prepared and warmed at 37°C. A 150 µL standard solution or sample was mixed with 2850 µL of FRAP reagent and kept in dark for 30 min. The absorbance of the produced ferrous tripyridyltriazine complex was recorded at 593 nm. The standard curve was linear between 25 - 800 µM Trolox. Additional dilution was made if the FRAP value measured was over the linear range of the standard curve. The antioxidant activities are expressed as pg Trolox equivalent cell<sup>-1</sup> (pg TE cell<sup>-1</sup>).

### 2.5. Total phenolic content

Total phenolics were extracted and measured according to Hajimahmoodi et al. (2012). In brief, 200 µL of standard solution or sample was mixed with 1.5 mL of Folin-Ciocalteu reagent which

was previously diluted tenfold with distilled water. After 5 min, 1.5 mL of 6% (w/v) sodium bicarbonate solution was added to the solution. The mixture was incubated for 90 minutes at room temperature and the absorbance was measured at 750 nm. The standard curve was constructed using gallic acid standard solutions (25-125  $\mu\text{g mL}^{-1}$  in 50% methanol). The results are presented as pg gallic acid equivalent cell<sup>-1</sup>.

## 2.6. Lipid peroxidation

For estimation of lipid oxidation, according to Hodges et al. (1999) with slight modification, 20 mL of each algal culture was centrifuged at 3000 g for 10 min. The pellet containing algal cells was homogenized in 80:20 ethanol:water, followed by centrifugation at 3000 g for 10 min. A 1 mL aliquot of sample was added to a 1 mL of either -TBA (thiobarbituric acid) solution comprised of 20% trichloroacetic acid or +TBA solution containing the above plus 0.65% TBA. Samples were then mixed vigorously, heated at 95° C in a water-bath for 25 min, cooled, and centrifuged at 5000 g for 10 min. Absorbances were read at 440, 532, and 600 nm. Malondialdehyde (MDA) equivalents were calculated as described by Hodges et al. (1999).

## 2.7. Ascorbate peroxidase (APX) activity

APX was extracted from *D. salina* as described previously (Nikookar et al. 2004). The activity of APX was assayed by following the decrease in absorbance at 290 nm ( $= 2.8 \text{ mM}^{-1} \text{ Cm}^{-1}$ ) (Amako et al. 1994).

## 2.8. Data analysis and statistics

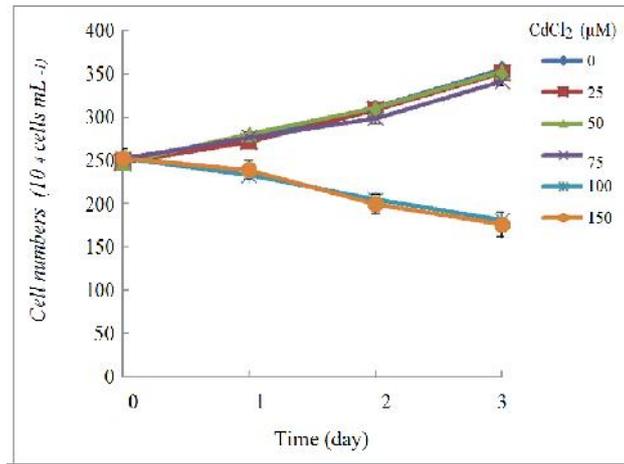
All results are the mean of three replicates  $\pm$  standard deviation (SD). Statistical analysis was performed using SPSS version 16.0. Duncan's multiple range tests was used to determine significant differences at  $p < 0.05$ .

# 3. RESULTS

## 3.1. Growth response to CdCl<sub>2</sub>

Figure 1 shows the growth responses of *D. salina* expressed as cell number ml<sup>-1</sup> of culture, as affected by different concentrations of CdCl<sub>2</sub> added to the growth culture at the exponential phase of growth. In the absence of CdCl<sub>2</sub> (the control) the number of cells ml<sup>-1</sup> of culture significantly ( $p < 0.05$ ) increase during the period of experiment. At 25, 50, and 75  $\mu\text{M}$  CdCl<sub>2</sub>,

the cell number determined at 24, 48, and 72 hrs. after the start of experiment was not significantly different from the cell number of control. At 100 and 150  $\mu\text{M}$   $\text{CdCl}_2$ , the cell number decreased significantly ( $p < 0.05$ ) during 72 h compared to the control, which is about 51% and 49.5% decrease in cell number, respectively.



**Fig. 1.** Cell number as affected by different concentrations of  $\text{CdCl}_2$  during three days period of algal exposure. Each value is mean  $\pm$  SD.

### 3.2. Effect of $\text{CdCl}_2$ on pigments content

The effects of different concentrations of  $\text{CdCl}_2$  on chlorophyll content expressed in terms of  $\mu\text{g ml}^{-1}$  of culture at 24, 48, and 72 hrs. after  $\text{CdCl}_2$  treatments are shown in Table 1. At control cultures chlorophyll content gradually increased over the experimental period. At 25, 50, and 75  $\mu\text{M}$   $\text{CdCl}_2$  changes in the chlorophyll content were not significantly different from the control. At 100 and 150  $\mu\text{M}$   $\text{CdCl}_2$ , chlorophyll content significantly decreased after 72 h of exposure to  $\text{CdCl}_2$ .

**Table 1.** Chlorophyll content ( $\mu\text{g mL}^{-1}$ ) as affected by different concentrations of  $\text{CdCl}_2$ 

Time (Day)	$\text{CdCl}_2(\mu\text{M})$					
	0	25	50	75	100	150
1	5.19±0.02	5.08±0.46	5.20±0.14	5.16±0.09	4.61±0.15*	4.36±0.12*
2	5.70±0.14	5.68±0.26	5.61±0.20	5.75±0.65	3.74±0.37*	3.65±0.32*
3	6.07±0.08	6.10±0.06	6.23±0.11	6.00±0.09	3.16±0.10*	3.00±0.12*

Values are mean  $\pm$  SD, n=3. In each row, significant differences at  $P < 0.05$  are shown by asterisks.

Total carotenoids as affected by  $\text{CdCl}_2$  are shown in Table 2. Total carotenoids per ml of control culture augmented with the time. Up to 75  $\mu\text{M}$   $\text{CdCl}_2$ , total carotenoids remained relatively unaffected and increase over three days period of experiment. In the presence of 100 and 150  $\mu\text{M}$   $\text{CdCl}_2$ , decrease in total carotenoids was observed on the first day of experiment with the constant for the second and third day of experiment.

**Table 2.** Carotenoids content ( $\mu\text{g mL}^{-1}$ ) as affected by different concentrations of  $\text{CdCl}_2$ 

Time (Day)	$\text{CdCl}_2(\mu\text{M})$					
	0	25	50	75	100	150
1	3.61±0.13	3.84±0.09	3.93±0.17	3.12±0.19	1.75±0.13*	1.76±0.02*
2	5.10±0.33	5.31±0.11	4.91±0.22	4.85±0.13	1.80±0.08*	2.00±0.05*
3	6.30±0.41	6.52±0.35	6.12±0.26	5.97±0.17	1.61±0.04*	1.69±0.06*

Values are mean  $\pm$  SD, n=3. In each row, significant differences at  $P < 0.05$  are shown by asterisks.

### 3.3. Effects of $\text{CdCl}_2$ on lipid peroxidation and antioxidant capacity of *D. salina*

When cells were treated with  $\text{CdCl}_2$  for 48 h at the exponential phase of growth, the degree of lipid peroxidation, expressed as nmol malondialdehyde (MDA) content per  $10^7$  cells was determined in Table 3. At 25, 50, and 75  $\mu\text{M}$   $\text{CdCl}_2$  of treated cells were close to MDA level in control cells. With increasing the content of  $\text{CdCl}_2$ , the level of MDA increased from 0.77 nmol  $10^7$  cell<sup>-1</sup> in the control, to 1.37 and 1.13 nmol  $10^7$  cell<sup>-1</sup> in the presence of 100 and 150  $\mu\text{M}$   $\text{CdCl}_2$ , respectively.

**Table 3.** Antioxidant activity (pg TE cell<sup>-1</sup>), total phenolic content (pg GAE, cell<sup>-1</sup>), MDA concentration (nmol per 10<sup>7</sup> cell), and APX activity ( O.D. min<sup>-1</sup> 10<sup>7</sup> cells) in *D. salina* as affected by different concentrations of CdCl<sub>2</sub> after 72 h of treatment. Values are mean ± SE, n=3. In each row, significant differences as compared to control (*P* < 0.05) are shown by asterisks.

	CdCl <sub>2</sub> (μM)					
	0	25	50	75	100	150
Total phenolic content (pg GAE, cell <sup>-1</sup> )	1.83±0.11	1.90±0.10	1.75±0.13	1.62±0.07	1.46±0.09*	1.43±0.12*
Trolox Equivalent (pg. cell <sup>-1</sup> )	10.3±0.81	10.0±0.74	10.12±0.44	10.04±0.65	8.00±0.37*	8.14±0.62*
MDA concentration (nmol per 10 <sup>7</sup> cell)	0.77±0.04	0.80±0.06	0.84±0.09	0.88±0.09	1.37±0.07*	1.13±0.05*
APX activity ( O.D. min <sup>-1</sup> 10 <sup>7</sup> cells)	0.38±0.02	0.41±0.05	0.35±0.03	0.45±0.02	0.59±0.01*	0.61±0.06*

Table 3 shows the APX activity expressed as O.D. min<sup>-1</sup> per 10<sup>7</sup> cell after 48 h exposure of algal cells to different concentrations of CdCl<sub>2</sub>. In the presence of 25 and 50 μM CdCl<sub>2</sub> the APX activity of cells was the same as the control. 75 μM CdCl<sub>2</sub> caused small but insignificant increase in the APX activity relative to control. At 100 and 150 μM CdCl<sub>2</sub> the APX activity of cells was higher compared to control.

Antioxidant (reducing) capacity of the algal cells treated with different concentrations of CdCl<sub>2</sub> was determined by the FRAP and the Folin-Ciocalteu methods (Table 3.). Significant decrease in antioxidant capacity was observed in the presence of 100 and 150 μM CdCl<sub>2</sub>.

#### 4. DISCUSSION

Aquatic ecosystems are susceptible to accumulating contaminants. Large quantities of heavy metals are release into aquatic ecosystems due to their widespread industrial use (Nriagu and Pacyna, 1988; Penuelas and Filella, 2002).

Despite an increasing literature on biochemical events in response to heavy metals stress in higher plants, little is known about unicellular algae. Microalgae play an important role in the equilibrium of aquatic ecosystems and represent highly suitable biological indicators of

environmental changes (Carfagna et al. 2013).

We use a strain of *Duanliella salina*, as a model system for physiological, biochemical and morphological studies on heavy metal stress response. The algal cells were treated for a short period (72 h) to different concentrations of CdCl<sub>2</sub>.

Cadmium is an important metal due to its industrial use but also one of the most dangerous metals because it is a highly mobile metal and highly soluble and toxic. So, a large number of publications have discussed the many aspects of stress induced by Cd and other metals in a wide range of species (Groppa et al. 2012; Tian et al. 2012; Martinez-Penalver et al. 2012; Mihucz et al. 2012).

Cd can affect cell biochemical mechanisms and structural aspects, for example, by reducing the control of the cell redox state, so inducing oxidative stress and disruption of membrane composition and function (Gratao et al. 2009; Gallego et al. 2012). Also, Cd can substitute for other metal ions (mainly Zn<sup>2+</sup>, Cu<sup>2+</sup>, and Ca<sup>2+</sup>) in metallo enzymes and shows a very strong affinity to biological structures containing –SH groups (Qian et al. 2009). Intense disturbance in the physiological processes of a plant, such as respiration, photosynthesis, water relations, and mineral uptake can be caused by Cd (Gill et al. 2012; Lopez-Chuken and Young, 2010).

Growth reduction in *D. salina* exposed to different CdCl<sub>2</sub> concentrations has been observed in other organisms including plants and algae (Lux et al. 2011; Kopittke et al. 2010; Sbihi et al. 2012). Cell division is one of the first algal processes affected by Cd. Cadmium inhibit cell proliferation and reduce the mitotic index (Rosas et al. 1984). The marine macroalga *Ulva fasciata* is tolerant to Cd ranging from 5-50 μM (Tzure-Meng et al. 2009). As shown in Fig. 1, in the absence of Cd, there was an increase in cell number during the 3-d period of experiment. Under the present experimental conditions *D. salina* is tolerant to Cd up to 75 μM. At 100 and 150 μM CdCl<sub>2</sub> decrease in cell number is due to increase in cell lysis and reduce in cell division of *D. salina*.

Decrease in chlorophyll content and total carotenoids in terms of μg ml<sup>-1</sup> algal culture is mainly due to the decrease in cell number ml<sup>-1</sup> of culture. Heavy metals often interfere in the biosynthesis of chlorophyll; reduce in the net photosynthetic rate and changes in enzymes activity which caused decreases in plant growth and productivity (Zhang et al. 2010; Macfarlane and Burchett, 2001).

A general toxicity mechanism for heavy metals is production of ROS. Generation of ROS and associated oxidative stress can cause the growth reduction of *D. salina*. A large number of reports have shown that Cd induces several ROS, such as the superoxide radical ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical ( $OH^{\cdot}$ ) (Benavides et al. 2005). Consequences of ROS formation include the gradual peroxidation of lipid structure (Baryla et al. 2000), oxidative DNA damage (Kasprzak 2002), and photosynthetic apparatus damage (Dewez et al. 2005; Vajpayee et al. 2005).

Lipid peroxidation which is considered as a biomarker of cellular damage, increased in *D. salina* treated with 100 and 150  $\mu$ M  $CdCl_2$ . So, oxidative stress has occurred during algal exposure to Cd. This is evident from the decrease in cell number and increase cell lysis in the presence of Cd. Lipid peroxidation induced by Cd was also evidenced in *Oryza sativa*, *Zea mays*, and *Dunaliella tertiolecta*, with the accumulation of malondialdehyde (Shah et al. 2001; Rellan-Alvarez et al. 2006; Zamani et al. 2009).

The deleterious effects resulting from the oxidative state of the cell can be mitigated by enzymatic and non-enzymatic antioxidant (Chaoui and El-Ferjani, 2005).

Several investigators have reported the increase in APX activity in response to heavy metals (Rellan-Alvarez et al. 2006). In marine macroalgae *Ulva fasciata* the activities of APX and catalase increased as Cd concentrations increased (Tzure-Meng et al. 2009). Although in *D. tertiolecta* activity of APX increased with increased  $HgCl_2$  concentrations (Zamani et al. 2009). APX reduces  $H_2O_2$  to  $H_2O$  using ascorbate as the reductant and decreases the damage caused to biomolecules by ROS.

Reducing substances, measured by electron transfer (ET) based assays, is one of the antioxidant defense substances which protect cell from oxidative stress (Zamani and Moradshahi, 2013). In the presence of 100 and 150  $\mu$ M  $CdCl_2$  antioxidant capacity of the algal cells was decreased significantly ( $p < 0.05$ ) compared to control. Under different stress conditions antioxidant defense mechanisms are usually used, so, increased antioxidant capacity is expected during exposure to heavy metals (Youn et al. 2011). It is possible that the reduction rates of low molecular weight antioxidant through their reaction with produced ROS are higher than their rates of generation. In addition to low molecular weight antioxidant, the expression of stress response genes may have been regulated by heavy metals. Antioxidative response of the marine macroalgae *Ulva fasciata*

Delile to cadmium stress showed that glutathione reductase and Fe-superoxidase activities and transcripts increased as Cd concentrations increased (Tzure-Meng et al. 2009). Effects of cadmium, on antioxidant enzymes (genes) expect further research.

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