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Humic Acid Mitigates Viability Reduction, Lipids and Fatty Acids of *Dunaliella salina* and *Nannochloropsis salina* Grown under Nickel Stress

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Abstract: The evaluation of Humic Acid (HA) to mitigate the viability reduction, lipids and fatty acids of *Dunaliella salina* Teodoresco and *Nannochloropsis salina* Hibberd cultivated in nickel contaminated media has been studied. The results revealed that toxicity of nickel decreased the cell viability to 40 and 30% of the control for *D. salina* and *N. salina*, respectively. Total fatty acid/lipid content has been reduced in both strains to about 28 and 20% of the control in the same order. Nickel induced alterations in the fatty acid composition in both investigated microalgae. An obvious increase was detected in the proportions of saturated profiles accompanied by a decrease in the unsaturated ones, except for C18:2 of *N. salina* which appeared to accumulate in the cells. Upon HA addition, the toxicity of nickel was significantly reduced ($p \leq 0.05$), cell viability enhanced to 25 and 15% for *D. salina* and *N. salina*, respectively. Total fatty acid/lipid content became 20 and 10% correspondingly. Concerning fatty acid composition, the response of the examined species appeared the same; both saturated and unsaturated fatty acid concentrations tend to be more or less around the control values. The study demonstrated a mitigation effect of HA towards the toxicity caused by nickel contamination. This mitigation has been explained through two ways. (a) The formation of HA-Ni^{2+}, a complex of high molecular weight and hence the metal was less bioavailable. (b) HA absorbed into the algal cell surface causing a protection from $NiCl_2$ stress.

Key words: Cell viability, humic acid, lipid, microalgae, nickel contamination

INTRODUCTION

Heavy metals showed to affect a wide range of algae cellular activities including cell viability (Franqueira *et al*., 2000) and membrane structure and properties (Guschina and Harwood, 2002). Toxic metals enter cells through the transport system of nutrient metals (Sunda and Huntsman, 1998). The transporter systems consist of proteins located in the external cell membrane containing different functional groups that interact with metal ions and permit their uptake into the cell interior (Campbell, 1995). Toxic effect often occurs when toxic metals displace nutrient from their metabolic sites (Sunda and Huntsman, 1998).

Heavy metals can cause membrane depolarization and acidification of the cytoplasm (Conner and Schimid, 2003) and in fact, membrane injury is one important effect of metal ions that may lead to disruption of cellular homeostasis (Sigaud-Kutner *et al*., 2003). Mining, smelting and waste industries liberate substantial amounts of heavy metals, including nickel, which are released into different habitats. Algae are the basis of the food web in all aquatic ecosystems they are responsible for about half of the O_2 production to the atmosphere (Gibson *et al*., 1990). Microalgae constitute the main food source for bivalve mollusks in all their growth stages, for different zooplanktons and larval stages of some crustacean and fish species. The nutritional value of an alga species is dependent on diverse of characteristics including shape, size, digestibility and toxicity (Sigaud-Kutner *et al*., 2003). The primary determination in establishing the food quality transferred to the other trophic levels of the food web appears to be the biochemical composition of the algae, fatty acids, sterols, amino acids, sugars, minerals and vitamins (Brown and Miller, 1992; Veloza *et al*., 2006). Stress treatments that affect an algal cell's biochemical composition will have a major impact on its food value.

Humic acids showed to play an important role in ameliorating heavy metal stress in microalgae (Kulikova *et al*., 2005) through a complexation mechanism by which it may influence the bioavailability of metal in naturalwaters (Koukal *et al*., 2003). Two microalgae have been chosen in this study; the chlorophyte *Dunaliella*

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salina Teodoresco, which is ingested by copepods (Veloza *et al*., 2006) and the eustigmatophyte *Nannochloropsis salina* Hibberd, which is ingested by *Brachionis plicatilis* and *Artemia* spp. (Brown *et al*., 1997; Brown, 2002). The two strains can accumulate large amount of lipids and have highly valuable compounds including different important fatty acids (Zittelli *et al*., 1999; Abd El-Baky *et al*., 2004).

Therefore, the aim of this study was to assess the mitigation effect of humic acid on *D. salina* and *N. salina* grown under nickel stress. The data provided on cell viability, total fatty acid/lipid content and fatty acid composition.

MATERIALS AND METHODS

Microalgae and culture conditions: The chlorophyte *Dunaliella* (Dunal) *salina* Teodoresco was obtained from the algal culture collection of the Faculty of Science, Alexandria University, Egypt. The cultures were grown axenically in MH medium according to Loeblich (1982). The eustigmatophyte *Nannochloropsis* (*Monallantus*) *salina* Hibberd was obtained from the Solar Energy Research Institute (SERI) Culture Collection in Golden, Colorado, USA. The cultures were grown axenically according to Boussiba *et al*. (1987). All cultures were grown in triplicate 1 L glass flasks at pH 7 and maintained at a temperature of 23±1°C. Cultures were continuously agitated by bubbling with sterile air enriched with 0.5% CO_2. Illumination was provided by fluorescent lamps with an irradiance of 300 μmol m^{-2} sec^{-1} at the surface of the cultures under a 12:12 h light: dark regime.

Extraction of Humic Acid (HA): From the top 6 cm of garden soil of Faculty of Science, Alexandria University; a sample was freshly collected and transported directly back to the algae laboratory, where it was immediately assayed for HA extraction according to Stevenson (1982). In brief, A 25 g sample of sieved uncontaminated garden soil was allowed to settle, the aqueous-phase material was decanted and discarded. Approximately 25 mL of water was added to the soil and the slurry was allowed to sit for 30 min. The pH was then adjusted to 7.0 with the addition of 1 M NaOH. The total volume of the slurry was brought to 250 mL with the addition of 0.1 M NaOH and the mixture was stirred for 24 h. The mixture was then centrifuged and the particle-free supernatant was adjusted to pH 1.0 by adding 6 M HCl with constant stirring. This resulted in the formation of a dark brown precipitate. The suspension was allowed to stand for 12 h and centrifuged to recover the precipitate. This precipitate was suspended in a solution of 0.1 M HCl and 0.3 M HF overnight to remove minerals impurities and then washed with distilled water to remove chlorides. The sample was centrifuged and the precipitated HA was freeze dried and stored in desiccator.

Infrared (IR) analysis of HA: A sample of HA was provided for IR analysis, to examine its validity, by mixing approximately 1 mg of dried HA with 100 mg of KBr. Infrared spectra were recorded using Perkin Elmer 1430 ratio recording infrared spectroscopy from 3650-700 cm^{-1} with an attached computer running. The result recorded as the mean of 10 sequential scans (Kansiz *et al*., 1999). Identification of major IR spectra of HA was based on the studies of Gaffney *et al*. (1996), Senesi and Brunetti (1996), Senesi *et al*. (1996) and Galli *et al*. (1997), relating to humic acids extracted from various sources.

Metal solution: A Stock solution of $NiCl_2$ was prepared in distilled water and stored at 4°C until use.

Preliminary experiments (unpublished data) have been performed to assess the appropriate dose-response for $NiCl_2$ and HA used: A series of $NiCl_2$ and HA concentration was individually provided (from 0.1 to 0.5 mg L^{-1}) at the end of exponentially grown cultures. A 0.5 mg L^{-1} of $NiCl_2$ was found to be the sub-lethal concentration after 48 and 72 h of incubation for *D. salina* and *N. salina*, respectively and hence it has been chosen as the dose-response after 24 h of incubation. On the other hand, a concentration of 0.2 mg L^{-1} HA was found to be the best appropriate dose enhancing the energetic potential for both microalgae.

Experimental design: At the end of the exponentially grown microalgae, the culture flasks were divided into four sets: the first set was the controlled cultures, the second was supplemented with 0.5 mg L^{-1} $NiCl_2$, the third was supplemented with 0.2 mg L^{-1} HA and the fourth was supplemented with 0.5 mg L^{-1} $NiCl_2$ plus 0.2 mg L^{-1} HA. After 24 h of incubation, the uptake of free Ni^{2+} ions was measured for the second and fourth culture sets using Atomic Absorption Spectrometry, Perken Elmer 2380, USA. The results obtained (unpublished data) showed a significant reduction effect of HA on the free Ni^{2+} ions uptake, 50 and 60% for *D. salina and N. salina*, respectively.

However, the different treated cultures were examined for their cell viability, total fatty acid/lipid content and fatty acid composition.

Cell viability: The cell viability was examined according to McGahon *et al*. (1995). One milliliter of a 1% w/v stock solution of trypan blue dye was added to 20 mL culture to yield a final concentration of 5×10^{-7} g mL^{-1}. The dye stains the nonviable cells blue. Measurements of cell

viability were conducted by counting cells on a haemocytometer (Fuchs-Rosenthal grid, 0.1 mm deep under the light microscopy, Olympus, Transmission, LM). Cell viability % = number of viable cells/number of total cells×100.

Determination of total lipids: For lipid extraction, the algal cells were harvested at the end of the logarithmic growth phase using the centrifuge JANETZKI T24, at 1000 g for 5 min. Cells were homogenized with chloroform-methanol (2:1 v/v) and refluxed for few minutes in order to inactivate the phospholipases. The extraction of total lipids and purification of the extracts were performed according to Bligh and Dyer (1959). The total lipid contents were quantified for different algae cultures.

Analysis of Fatty Acid Methyl Esters (FAME): The total lipid fractions were subjected to the saponification and then converted into methyl esters using the procedure of Radwan (1991). FAME were quantified and identified on a Shimadzu Gas Liquid Chromatography (GLC), equipped with a flame ionization detector with packing column material Hp-5. The carrier gas was nitrogen and the short speed was 5 mm min^{-1}. Identification of FAME was carried out by comparing their retention times with those of standards. Quantification was based on the internal standard method.

Statistical analysis: Data were analyzed by means of two-way analysis of variance (ANOVA), using COSTAT 2.0 statistical analysis software. Means were tested with least square difference (LSD), where the difference of $p \leq 0.05$ was significant. The mean value of triplicate data and the Standard Deviations (SD) were also calculated.

RESULTS AND DISCUSSION

IR analysis of HA: The attribution of the main absorption bands (Table 1) indicated the presence of a variety of functional groups, the major of them being carboxylic acids, phenols, alcohols, amides, amines, aliphatic chains, aromatics, proteins, carbohydrates and fatty acids. The results confirmed the validity of HA used.

Influence of HA on the algae cultures: The results showed that HA have no effect on the lipid content or fatty acid composition of the investigated microalgae and hence no represented data is concerned.

Influence of $NiCl_2$ and HA on the cell viability of the studied microalgae: Present results demonstrated a significant reduction ($p \leq 0.05$) in the cell viability caused by the exposure of microalgae to 0.5 mg L^{-1} $NiCl_2$, the reduction percent was 40 and 30% for *D. salina* and *N. salina*, respectively (Fig. 1). Addition of 0.2 mg L^{-1} HA significantly reduced this toxicity to 25 % for *D. salina* and 15% for *N. salina* comparing to the control cultures.

Table 1: Attribution of bands in IR analysis of HA extracted from garden soil

Frequency (cm^{-1})	Attribution (References)
~3500-3300	O-H vibration of the hydroxyl groups of phenols, alcohols and carboxyl functions and N-H vibrations from amides and amines.
~2925	Symmetric CH stretching in-CH3 and-CH2-of aliphatic chains.
~2840	Asymmetric CH stretching in-CH3 and-CH2-of aliphatic chains.
~1650	C = O stretching of primary amides associated with proteins.
~1540-1510	N-H deformation in the secondary amides associated with proteins.
~1455	Asymmetric CH3 stretching and asymmetric CH2 stretching of proteins.
~1440	Aliphatic C-H deformation of structures fatty acids.
~1398	CH3 symmetric and CH2 symmetric of proteins and symmetric C-O stretching of COO^- groups.
~1380	O-H deformation, C = O stretching of phenols, anti-symmetric COO-stretching and aliphatic C-H deformation.
~1260	C-OH stretching of aromatic groups and-C-O-C stretching of aryl ethers and phenols.
~1080	Symmetric P = O stretching of the phosphodiester backbone of nucleic acids.
~900	C-O-C stretching of carbohydrates.

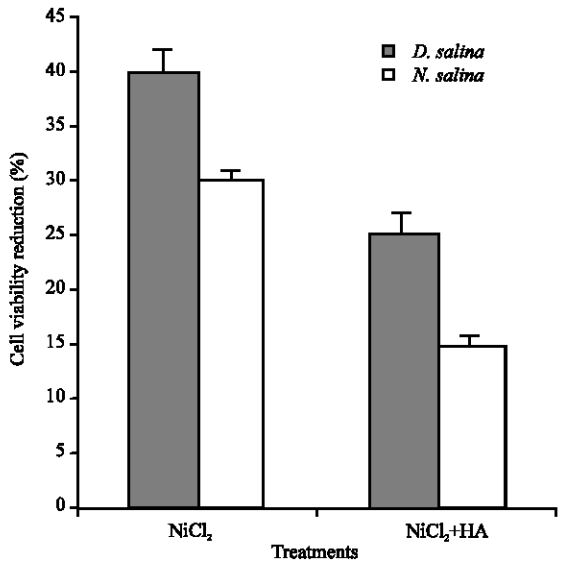

Fig. 1: Cell viability reduction percent of *D. salina* and *N. salina* grown under $NiCl_2$ and $NiCl_2$ + HA

Influence of $NiCl_2$ and HA on the total fatty acid/lipid content of the studied microalgae: Total fatty acid/lipid content (TFA, μg mg^{-1} lipid) were recorded for the two species at Fig. 2. In *D. salina*, the deteriorating percent

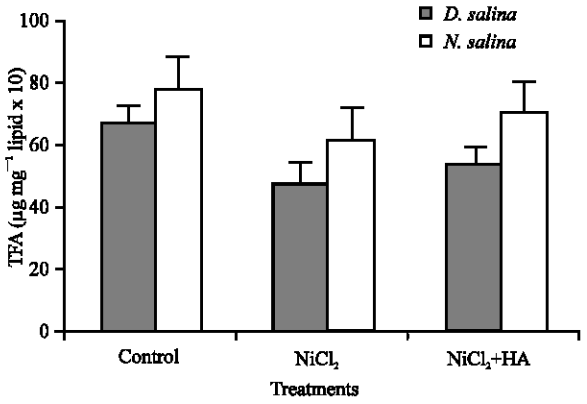

Fig. 2: Total fatty acids/lipid content of *D. salina* and *N. salina* grown under different treatment conditions

in cells grown in $NiCl_2$ contaminated cultures was approximately 28% of control. While in the cells grown under a mixture regime of HA and $NiCl_2$, the reduction was approximately 20%. In *N. salina*, the percent of total fatty acid/lipid content decreased in Nickel contaminated cultures by approximately 20% of control while in the cells grown with $NiCl_2$ and HA, the decrease percent was 10% only.

Influence of $NiCl_2$ and HA on the fatty acid composition of the studied microalgae: Fatty acid composition of the studied strains recorded in Tables 2 and 3. In *D. salina*, the major fatty acids were concentrated among C16 profiles, C14:1 and C18:3. Other profiles (C 14:0, C18, C20, C22 and C24 fatty acids) were detected in minor amounts (<10%). The proportions of fatty acid composition were markedly altered in cells grown in $NiCl_2$ contaminated cultures. A remarkable increase in saturated acids (C14:0, C16:0, C20:0 and C22:0) was observed. However, there is a decrease in the proportions of unsaturated profiles particularly C16:1, C16:4, C18:3, C22:4 and C24:1. Fatty acids subjected to variation in *N. salina* were C14:0 and the profiles of C16, C18 and C20. A marked increase in the proportions of saturated profiles accompanied by a decrease in the unsaturated ones except for C18:2 which inclined to accumulate in cultures treated with $NiCl_2$. However, in cultures supplemented with $NiCl_2$ and HA, the examined microalgae showed a similar response, where their content of saturated and unsaturated fatty acid concentrations tends to be more or less around the control values.

Cell viability is a function of the physiological state of an examined cell (Harrison, 1988). In this contribution we demonstrated the toxic effect of $NiCl_2$ on the cell viability of the two investigated microalgae. Our results agreed with those obtained by Rai and Raizada (1987) and Rai *et al.* (1990) on *Nostoc muscorum* and by Cid *et al.* (1998) on *Phaeodactylum tricornutum*. The reduction of cell viability, as a result of cytotoxicity, is due to the incapacity to finish cell division (Franqueira *et al.*, 2000).

Table 2: Fatty acid composition of *D. salina* grown under different treatments (control, 0.5 mg L^{-1} $NiCl_2$ and 0.5 mg L^{-1} $NiCl_2$ + 0.2 mg L^{-1} HA), data expressed as wt % of fatty acid fraction

Treatments			
*FAME	Control	$NiCl_2$	$NiCl_2$+HA
C14:0	0.1±0.1	1.1±0.1	0.6±0.4
C14:1	11.2±20	10.1±10	12.0±10
C16:0	13.0±10	19.2±30	11.7±30
C16:1	14.0±20	8.4±0.1	13.6±20
C16:4	22.2±30	14.1±10	21.5±10
C18:1	2.2±0.1	1.6±0.1	2.1±10
C18:2	2.6±10	1.8±0.1	3.0±0.1
C18:3	15.0±30	11.2±1.1	13.9±10
C20:0	6.8±10	15.0±20	6.0±0.2
C20:4	4.0±0.1	3.2±10	6.1±0.2
C22:0	3.1±0.1	11.2±20	2.9±0.1
C22:4	2.8±0.1	1.6±0.1	3.0±0.3
C24:1	3.0±10	1.5±0.1	3.6±0.1

*Fatty acid methyl esters

Table 3: Fatty acid composition of *N. salina* grown under different treatments (control, 0.5 mg L^{-1} $NiCl_2$ and 0.5 mg L^{-1} $NiCl_2$ + 0.2 mg L^{-1} HA), data expressed as wt% of fatty acid fraction

Treatments			
*FAME	Control	$NiCl_2$	$NiCl_2$+HA
C14:0	3.1±0.1	4.6±0.1	2.9±0.1
C16:0	2.8±0.1	5.5±2	9.0±20
C16:1	10.3±1	5.1±1	8.7±10
C16:2	6.5±0.1	3.5±1	7.3±20
C16:3	3.5±0.1	0.5±0.1	4.1±0.1
C18:0	3.3±1	7.3±2	3.1±0.1
C18:1	10.2±2	8.2±1	9.2±20
C18:2	6.2±1	12.4±3	5.9±10
C18:3	5.4±1	0.4±0.1	5.1±10
C20:0	3.2±0.1	8.8±1	4.2±10
C20:1	9.4±2	7.4±1	9.6±0.2
C20:2	8.7±1	8.1±2	9.3±0.3
C20:5	22.0±1	18.2±3	21.6±20

*Fatty acid methyl esters

However, upon addition of HA, cell viability improved by approximately 37% for *D. salina* and 50% for *N. salina*. In view of the fact that presence of HA decrease the uptake of the heavy metal causing reduction in their toxicity (Vigneault and Campbell, 2005).

Indeed, under Nickel stress a significant decrease ($p \leq 0.05$) of total fatty acid/lipid content has been observed in *D. salina* and *N. salina*. As well known, heavy metals are involved in many ways in the production of activated oxygen species that actively induce peroxidation of membrane lipid. A decrease of enzymic free radical scavengers caused by heavy metal stress may contribute to the shift in the balance of free radical metabolism towards accumulation, leading further to more breaks down of membrane lipids (Ouariti *et al.*, 1997). Therefore, we propose that the loss of lipids in both microalgae treated with $NiCl_2$ may be related to an enhanced rate of catabolism and/or to the suppression of lipid biosynthesis.

Present results revealed a significant alteration ($p \leq 0.05$) of fatty acid in both investigated microalgae grown under $NiCl_2$. The decrease in the proportions of unsaturated profiles suggesting that Nickel treatment induced an alteration in the fatty acid desaturation processes. The accumulation of C18:2, as explained by Ouariti *et al.* (1997), in *N. salina* indicated an alteration in the ratio of products from the fatty acid synthase. However, upon HA addition, the saturated and unsaturated fatty acid concentrations tend to be more or less around the control values in both studied microalgae. Suggesting an improvement effect of HA to fatty acid pool and hence an improvement in the cell permeability (Franqueira *et al.*, 2000).

Heavy metals noted to change fatty acid biosynthesis in plants (Jones and Harwood, 1993). The decrease in unsaturated fatty acid levels might be related to direct reaction of oxygen free radicals with unsaturated lipids (Ouriti *et al.*, 1997). As postulated by Guschina and Harwood (2002) that sensitivity of metabolic pathways can depend on altered gene expression. The internal metal concentrations in the vicinity of the enzymes concerned, as well as the interactions of membrane PUFAs in generating peroxidation products.

In this study, the modulation of lipid content and the fatty acid composition upon the addition of HA is clearly demonstrated. Humic substances can be considered as an environmental modulator mitigating the harmful consequences of stress factors (Kulikova *et al.*, 2005). The mechanism of HA to modulate the harmful effects of heavy metals in microalgae has been studied by many authors; Koukal *et al.* (2003) demonstrated that HA decrease the amount of free metal ions through metal-HA complex formation which in return shielded the algal cells from the damaging. Xue and Sigg (1990) postulated that both HA and algal surfaces carry out a net negative charge and hence both show high affinity for cationic metal species. The reduced bioavailability of Nickel in the presence of HA is believed to be due to competition for metals between complexing sites on HA and algal surface (Sunda and Huntsman, 1998). HA have a surfactant-like structure; they contain both hydrophobic domains (hydrocarbon chains) and hydrophilic moieties (carboxyls, hydroxyls, etc.) (Ghabbour and Davies, 1999). Due to this amphiphilic character, HA have been reported to adsorb on a large number of natural surfaces including diverse biological membranes (Campbell *et al.*, 1997). The adsorbed HA prevent metals from getting into the cell interior, presumably by reducing cell surface site availability to toxic metal (Sunda and Huntsman, 1998). Knauer and Buffle (2001) reported the adsorption of HA on algal cell surface may take place via hydrophobic interactions; for example, lipids and proteins in the cell wall and on the plasma membrane containing hydrophobic moieties that could interact with the hydrophobic domains of macromolecules such as HA. However, it might also take place via cohydrogen bonding, which requires coadsorption of H^+ to overcome electrostatic repulsion (Campbell *et al.*, 1997). Many different techniques following changes in the electrophoretic mobility of individual cells (Vigneault *et al.*, 2000) as well as transmission electron microscopy (Campbell *et al.*, 1997) have successfully employed to demonstrate the adsorption of humic substances on biological surface.

D. salina and *N. salina* differ in their response towards nickel stress as well as the outcome of Ni-HA interaction effects, the latter being more resistant. As well known, *D. salina* is characterized by the absence of the rigid cell wall, whereas *N. salina* is characterized by having a cell wall. In this respect, Bhatnagar and Bhatnagar (2000) reported that the most important feature providing protection to the alga is the cell wall. Previous studies demonstrated that *Nannochloropsis* is a tolerant organism to different stresses; Moreno-Garrido *et al.* (1998) described *N. gaditana* by having a hardness cell wall and it has a high resistance to the drastic treatments. The same conclusion was demonstrated for both *N.* sp. treated with the herbicidal compound DCMU (Gonen-Zurgil *et al.*, 1996) and *N. salina* treated with the aqueous diesel fuel pollution (Mohammady *et al.*, 2005).

CONCLUSION

Overview of present results indicated that $NiCl_2$ significantly reduced the viability and total fatty acids/lipid content of the two examined microalgae. However, *N. salina* appeared to be the most tolerant species. $NiCl_2$ induced alterations in fatty acid composition in both examined species; generally, an increase in the proportions of saturated profiles was detected accompanied by a decrease in the unsaturated ones. Based on the previous studies, addition of HA mitigates the destructive effects caused by $NiCl_2$ through two ways: a- The formation of HA-Ni^{2+}, a complex of high molecular weight and hence the metal was less bioavailable. b- HA absorbed into the algal cell surface causing a protection from $NiCl_2$ stress.

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