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ORIGINAL ARTICLE

Physiological Response of the Green Algae *Ulva lactuca* (Chlorophyta) to Heavy Metals Stress

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To monitor physiological changes induced by heavy metals contamination on the marine algae *Ulva lactuca* (Chlorophyta), laboratory experiments were performed. Physiological effects of four heavy metals (Cu, Pb, Zn and Cd ions) on *U. lactuca* were assessed 5 days after exposure under laboratory conditions. Fourier transform raman spectroscopy (FT-Raman) technique was also applied as support for physiological study. Our data showed that the specific growth rate (SGR%), chlorophyll (Chl *a* & *b*), total chlorophyll and carotenoids (Car) pigments decreased significantly in algal thalli under heavy metals stress. This phenomenon was more pronounced with Pb treatment compared to the other tested metals. In addition, morphological changes due to heavy metals treatment were recorded by observed cellular damages under the above mentioned metals. FT-Raman technique showed that the C-H, C=O, CH_2 and C-O-C groups were mainly involved in heavy metals absorption. Moreover, Pb ions showed the highest toxicity against *U. lactuca* studied by showing the highest decline in the above mentioned physiological parameters.

Key words: Ulva lactuca, Heavy metals, Pigmentations, Physiological changes

Living organisms such as macroalgae could be taken into account as a useful tool to monitor heavy metals pollution by recording physiological alterations. Earlier research stated the important role of macro – and microalgae in water bioremediation (Wang *et al.,* 1998; Gerofke *et al.,* 2005). Algae are able to uptake pollutants from their aquatic environment and bioaccumulate and biotransform organic matters and immobilize inorganic elements to make them less toxic. Detection of pollutants and their toxicity (physiological and biochemical markers) in photosynthetic living organisms could be considered as an early indicator of potential hazard in aquatic systems. Previously, Cairns and Van der Schalie (1980) reported that "biological monitoring or biomonitoring can be defined as the systematic use of biological response to evaluate changes in the environment, with the intent of establishing a quality control program". The physiological response of algae towards chemical pollutants was extensively investigated in many reports (Amado Filho *et al.,* 1996, 1997; Lewis *et al.,* 1998; Collén *et al.,* 2003; Torres *et al.,* 2008; Unal *et al.,* 2010; Jiang *et al.,* 2013).

It is well established that, some trace elements such as copper (Cu), zinc (Zn) and iron (Fe) in low concentrations are essential for catalyzing enzymatic reactions in living organisms. Whereas, other elements such as lead (Pb), mercury (Hg) and cadmium (Cd) could be associated as cofactors for activation of their enzymatic systems (Torres *et al.,* 2008; Manoj and Padhy 2013). Torab-Mostaedi *et al.* (2013) stated that among heavy metals, lead (Pb), cadmium (Cd), copper (Cu), zinc (Zn), nickel (Ni), chromium (Cr) and mercury (Hg) were the most investigated in ecosystem pollution.

It has been demonstrated that heavy metals stress provokes oxidative stress leading to the induction of reactive oxygen species (ROS) (Manoj and Padhy 2013). This effect caused DNA damages, proteins and lipids deformation and since cell death (Collén *et al.,* 2003; Manoj and Padhy 2013).

Among living organisms, macroalga *Ulva lactuca* could be considered as a useful bioindicator of heavy metals pollution due to their simple morphology, quick growth and their ability to accumulate high amounts of different chemical pollutants in their tissues. Thereby, they were extensively used for contamination studies in coastal ecosystem (Lu *et al.,* 2008; Nielsen *et al.,* 2012; Chakraborty *et al.,* 2014).

Among the seaweeds, *U. lactuca* was the most abundant marine algae along the coast regions of Lattakia-Syria. Indeed, it has the ability to reflect the levels of trace elements; thereby it can serve as a useful bioindicator for pollution in marine environment. So, they have been selected for this study. Therefore, toxicity of 4 metal ions (Cu, Pb, Zn and Cd ions) has been examined in the green macroalgae *U. lactuca*, by monitoring physiological alterations induced by the previous ions under laboratory conditions.

MATERIALS AND METHODS

Collection of algae samples

U. lactuca algal samples were collected along the Mediterranean Sea of the Syrian coast. Sampling was carried out from 35°33′790′′N longitude, 35°43′996′′E latitude at 4 km North Lattakia - Syria. Collection of only individuals with the similar size samples was performed by hand with disposable gloves. Algae were washed with seawater where they were collected and then transported within a flask containing 5 l seawater.

Algae cultivation and application of pollution

Algae samples were washed twice upon their arrival to laboratory with autoclaved artificial seawater ASW (500 mM NaCl, 10 mM KCl, 30 mM $MgSO_4$, 10 mM $CaCl_2$ and 10 mM Tris-HCl at pH 7.8) medium as previously described by Unal *et al.* (2010).

Then, they were divided into fresh flasks with a fresh ASW as previously described and kept under controlled laboratory conditions (Temperature of 20˚C, photoperiod of 12/12 h dark/light and illumination of 2950 Lux (~48.7 µmol photons $m^{-2}s^{-1}$) for 3 days before heavy metals stress application.

The mentioned ASW was considered as a control. Whereas, chemical stress was applied by adding 18.2 mg/l of Pb^{2+}, 5.8 mg/l of Cu^{2+}, 10.5 mg/l of Cd^{2+} and 9,9 mg/l of Zn^{2+} (Standard solution (1000 mg/l) from Fisher Scientific – UK, under their nitrate forms) for each treatment with three replicates/treatment. Experiment was performed in flasks with 300 ml ASW (as control) or with heavy metals (as stressed plants). The same previously described controlled conditions were maintained during the experimental stress application. Five days later, algae were harvested for physiological study. Our choice of ASW instead of seawater, is that with ASW no risk of chemical contamination due to their known and defined composition. Indeed, ASW works well under conservative operations (Kaladharan 2000).

Cell viability

Microscopic observation was performed to monitor pollution exposure in *U. lactuca* green algae. Algal lamina was visualized by Olympus DP70 (40x/0.65 Ach Ph2/0.17) microscopy.

Specific growth rate (SGR%)

The experiment was conducted in triplicates for 5 days. Algal specific growth rate (SGR%) was calculated in both control and stressed conditions according to Nielsen *et al.* (2012) as follows:

$$(SGR\%) = 100 \times (\ln (W_t/W_0))/t$$

Where W_0 was the initial biomass and W_t corresponded to the biomass after t days.

Extracted pigments

Chlorophyll (Chl) and carotenoids (Car) pigments were extracted in 80% acetone solvent. A hundred mg of thalli for each treatment were grind and 5 ml of acetone were added; then samples were kept in dark conditions at 4˚C for 24 h. Samples were centrifuged at 1400 g/ 2 min. Then, the extracts were filtered with Whatman filter papers; their absorbance was measured at 470, 645 and 662 nm. Chl *a* & *b*, total Chl and Car content was estimated as previously described by Lichtenthaler and Wellburn (1985).

$$\text{Chl } a \text{ (mg / g FW)} = 11.75\, A_{662} - 2.35\, A_{645}$$

$$\text{Chl } b \text{ (mg / g FW)} = 18.61\, A_{645} - 3.960\, A_{662}$$

$$\text{Car (mg / g FW)} = (1000\, A_{470} - 2.270 \text{ Chl } a - 81.4 \text{ Chl } b) / 230$$

$$\text{Tot Chl (mg / l)} = 20.2\, (A_{645}) + 8.02\, (A_{662})$$

Osmotic potential

One hundred mg of thali were cut and transported immediately to 2 ml Eppendorff with 2 ml dH_2O. Algae samples were ground and the tubes were centrifuged at 1400 g for 2 min, then 50 µl of supernatants were transferred to a new fresh 1.5 ml Eppendorff one. The osmotic potential was measured using a micro-osmometer (Osmomette) apparatus.

Batch biosorption experiment

Ion concentration and contact time detection was performed in batch experiment to investigate metal ions biosorption. For this purpose, ion concentration was measured before experiment starting (as an initial concentration) and after 5 days (end of experiment as a final concentration) using Atomic absorption spectrometry (AAS) technique. Whereas, for contact time assay, a batch test has been performed at room temperature of 20 °C, by mixing 0.25 g of algae biomass with a solution supplied with 5 mg/l of each Pb, Cu, Cd and Zn heavy metals in a final volume of 40 ml solution pH was adjusted to be 7. All metal solutions were prepared with ddH_2O. Algae samples were mixed with the previous solutions separately with various time intervals (15 min, 30 min, 45 min, 60 min, 120 min, 180 min and 240 min). Then they were filtrated using Watman filter paper 0.45 µm. The filtrated phase was used as template to determine algae biomass capacity to remove each mentioned ions from aqueous solution.

Whereas, for the effect of ion concentration on performance of algae to remove each ion, different concentrations from each above ions (1.5, 3.75, 7.5, 11, 15, 22.5, 26 and 30 mg/l) were tested during 2 h with 0.25 g of algae biomass.

In this respect, the algal potential removal (q) from aqueous solution was calculated as following:

$q = (c_0 - c_t) * V/W$

While, the absorbed (R%) of each ion was estimated as following:

$R = (c_0 - c_t)/ c_0 * 100$

Where c_0 the initial ion concentration (mg/l); c_t the residual ion concentration (mg/l); V volume of aqueous solution (l) and W weight of algae biomass (g).

Fourier transforms raman spectroscopy (FT-Raman) technique

Algal pigments that were previously extracted and filtrated were used as template for FT-Raman measurement using NXR FT-Raman (Thermo, USA) instruments.

Statistical analysis

Statistical analyses were performed using Statview 4.5 (Abacus 1996) statistical package at the 5% significance level ($P = 0.05$). Data were subjected to analysis of variance (ANOVA) for the determination of differences in means between tested algae plants of each heavy metal applied. Differences between means were tested for significance by Fisher's least significant difference (PLSD) test. Data are expressed as mean of three replicates.

RESULTS

The toxicant impact of heavy metals stress on algae tissues could be included, chloroplasts alterations, color alteration (chlorosis) and cellular deformation and death compared to their respective control (Fig.1). Microscopy test was performed to monitor pollution exposure in *U. lactuca* green algae.

Algal lamina was visualized by Olympus DP70 (40x/0.65 Ach Ph2/0.17) microscopy. Microscopy test analysis revealed some morphology changes in thali cells under heavy metals treatment.

Indeed, observed inhibition in different physiological parameters tested in this investigation was recorded. In this respect, a reduction in algal specific growth rate (SGR%) (Fig. 2), pigmentation including Chl *a* (Fig. 3a); Chl *b* (Fig. 3b), Car (Fig. 3c) and total Chl (Fig. 3d) and also in potential osmotic was observed (Fig. 4). Algae capacity to remove the four mentioned ions were also evaluate in batch biosorption experiments. The effect of ion concentrations on *U. lactuca* biosorption of the examined heavy metals is illustrated in Fig. 5. Our data showed that algae biosorption potent (q) increase as ion concentration increase (Fig. 5a). Also, It was noticed that R% capacity decreases as ion concentration increase during of 2 h of contact time (Fig. 5b).

As for contact time, our data demonstrated that algae followed the same trends of biosorption regardless examined contact time. For this reason, data concerning this parameter not presented herein.

Otherwise, FT-Raman technique was employed to characterize different peaks related to the functional groups yielded by heavy metals absorption of *U. lactuca* green algae (Fig. 6). In this respect, no differences were observed between control and treated plants spectra.

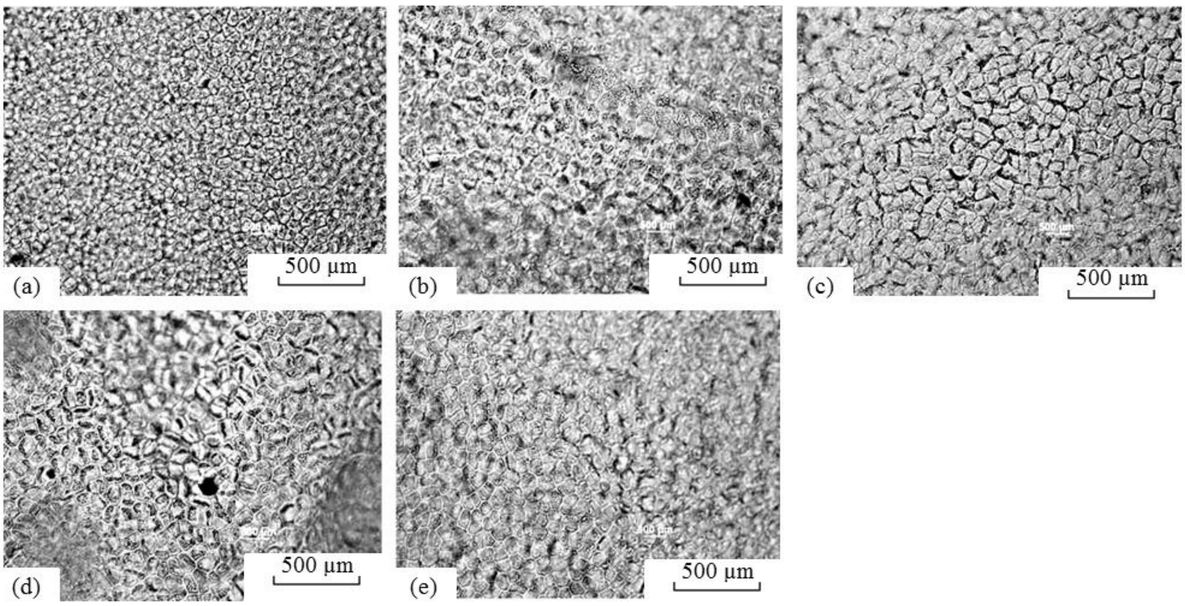

Figure 1. Microscopy test shows *U. lactuca* morphological alterations after 5 days exposure to heavy metals (Pb, Cu, Cd and Zn) stress. (a): Control; (b): Pb treatment; (c): Cu treatment; (d): Cd treatment and (e): Zn treatment.

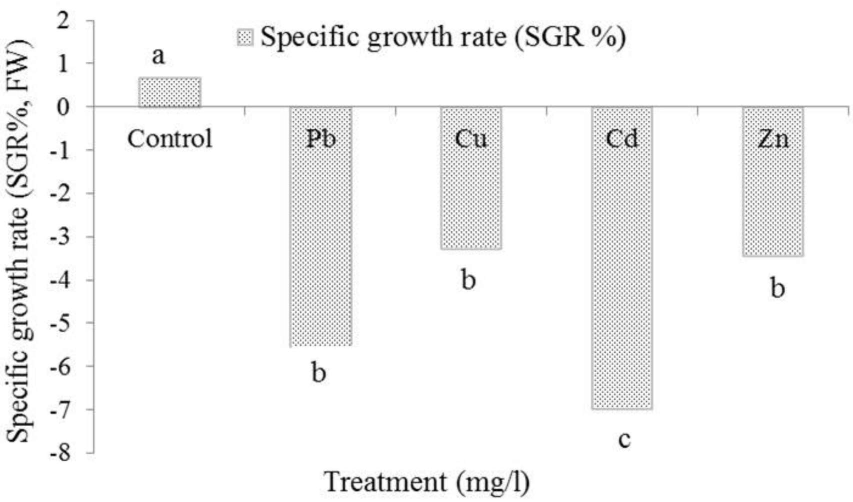

Figure 2. *U. lactuca* algal specific growth rate (SGR%) 5 days after heavy metals stress in both control and stressed conditions (n = 3)

Notes. Figures sharing the same lowercase letter are not significantly different at p = 0.05 probability by Fisher's PLSD test. LSD0.05 Specific growth rate / treatment: 2.613.

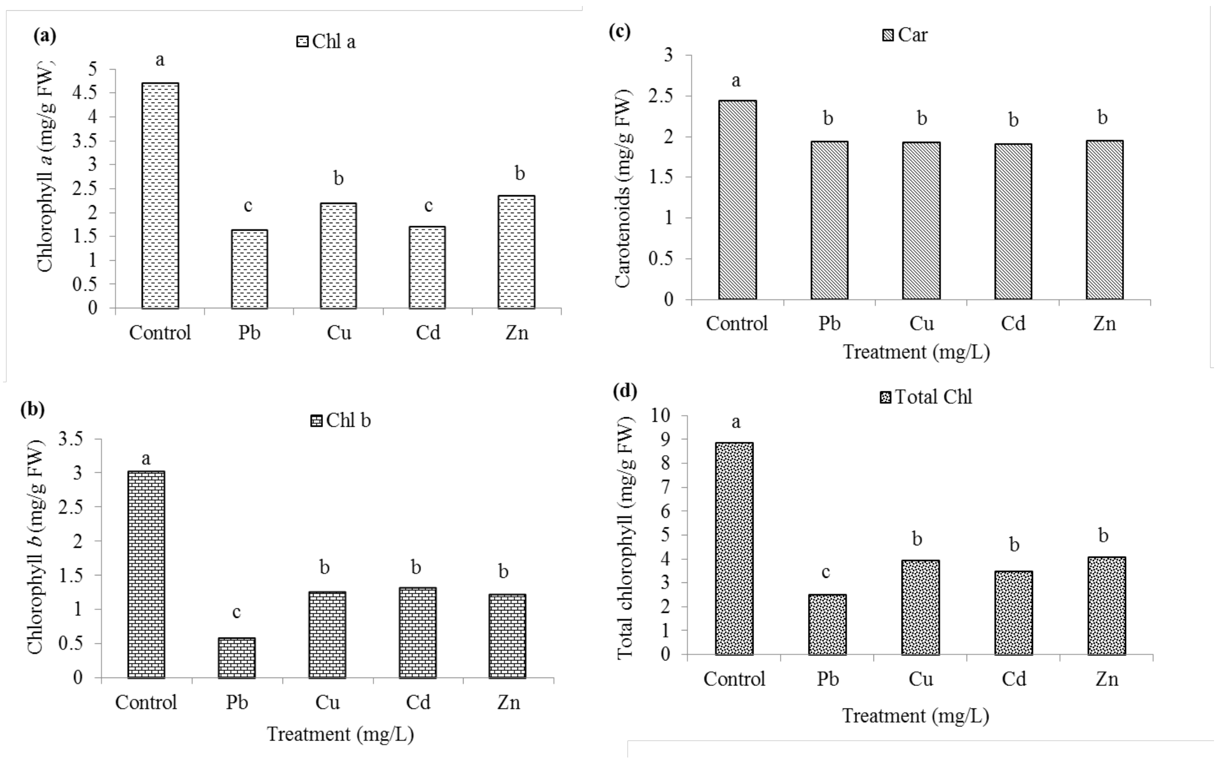

Figure 3. Pigmentation content of *U. lactuca* under control and heavy metals stress. (a): Chlorophyll *a*; (b): Chlorophyll *b*; (c): Carotenoids and (d): Total chlorophyll (mg/ g fresh weight) (n = 3)

Notes. Figures sharing the same lowercase letter are not significantly different at *p* = 0.05 probability by Fisher's PLSD test. $LSD_{0.05}$ Chl *a* / treatment: 0.602; $LSD_{0.05}$ Chl *b* / treatment: 0.258; $LSD_{0.05}$ Car / treatment: 0.086 and $LSD_{0.05}$ Total Chl / treatment: 0.905.

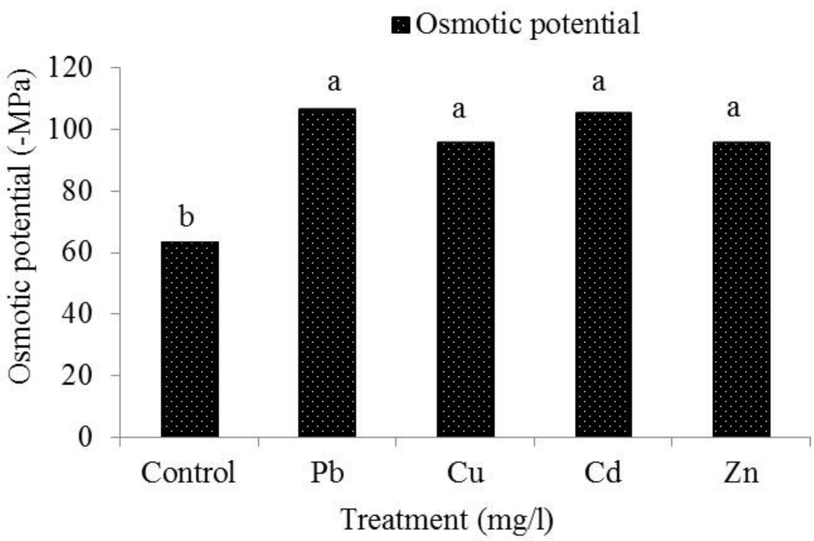

Figure 4. Osmotic potential (−MPa) of *U. lactuca* under control and heavy metals stress after 5 days exposure to heavy metals stress (n = 3)

Notes. Figures sharing the same lowercase letter are not significantly different at p = 0.05 probability by Fisher's PLSD test. LSD0.05 total osmotic potential / treatment: 11.468.

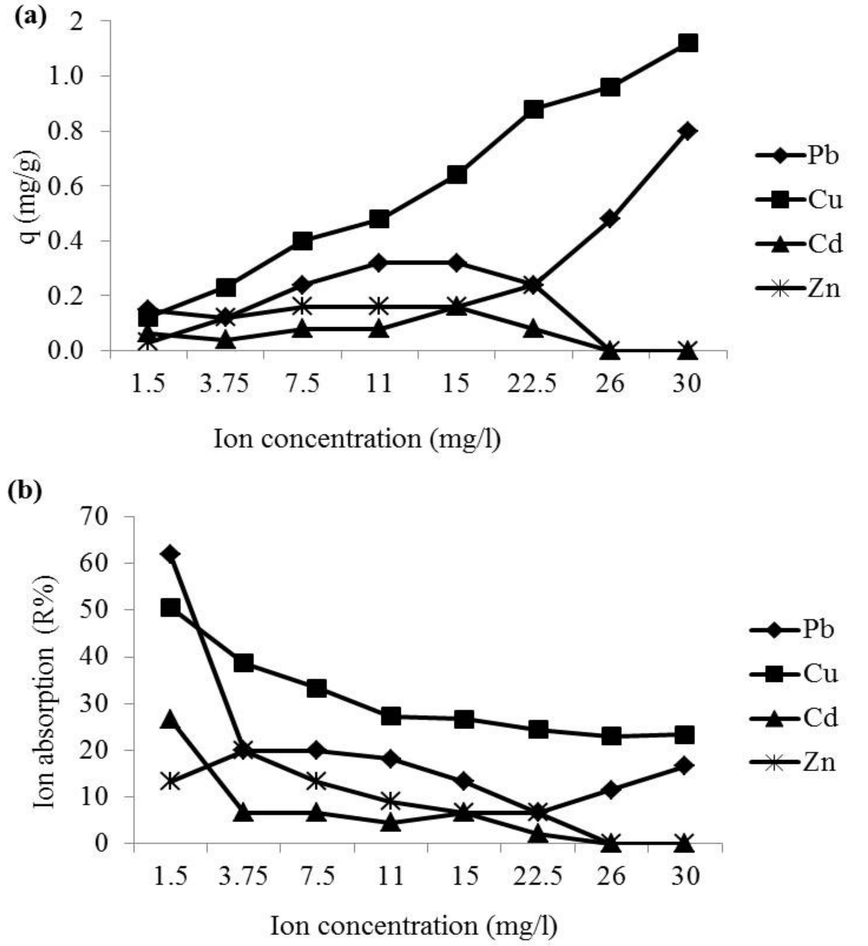

Figure 5. Effect of ion concentration on the algal potential removal (q) (a) and biosorption (R%) (b) of *U. lactuca* exposed to different heavy metals (Pb, Cu, Cd and Zn); (ion concentration of 1.5, 3.75, 7.5, 11, 15, 22.5, 26 and 30 mg/l; 0.25 g of algae biomass; Tm = 20 °C) (n = 3)

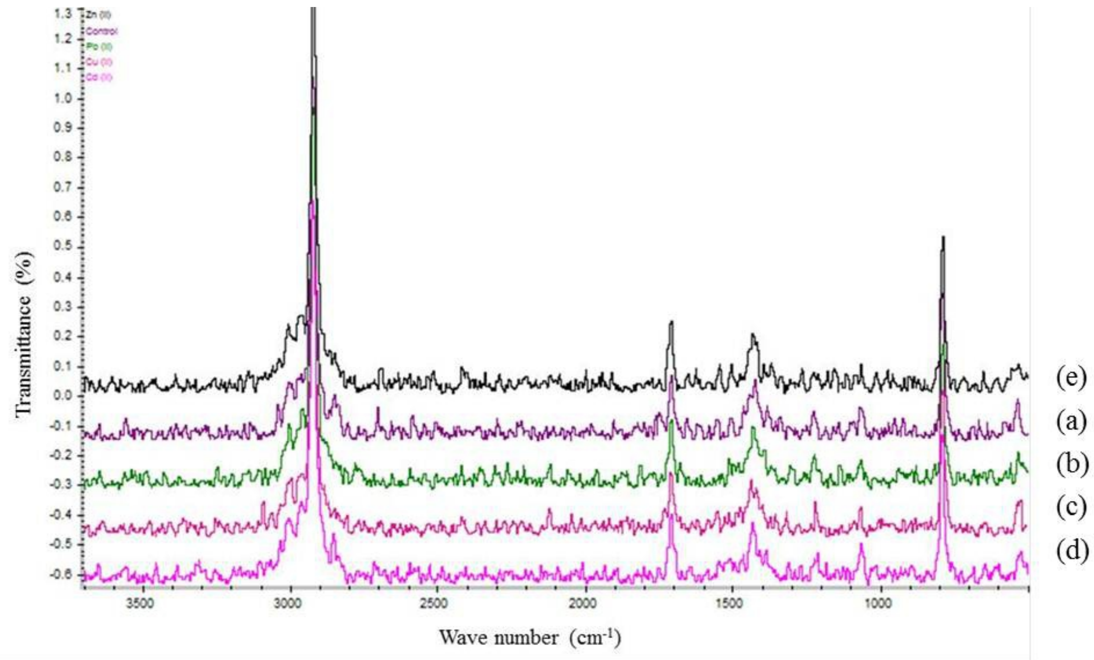

Figure 6. FT-Raman spectra of U. lactuca exposed to different heavy metals (Pb, Cu, Cd and Zn) stress. (a): Control; (b): Pb treatment; (c): Cu treatment; (d): Cd treatment and (e): Zn treatment

DISCUSSION

Exposure of algae to heavy metals stress induced chlorosis of algae tissues. Our data revealed some observed cellular morphological alterations with heavy metals exposure (Fig. 1). Similar findings were also reported in the response of *U. lactuca* investigation to different concentrations of Chromium VI (Unal *et al.,* 2010).

Toxicity of heavy metals treatment induced chlorosis of algae thalli in different manner according to examined ion. Earlier, Amado Filho *et al.* (1996 & 1997) reported that in *Padina gymnospora* brown algae, Zn ion was less toxic than Cu. Where their inhibition growth was observed with 0.020 mg/l of Zn for 7 days. These observations indicated that photosynthetic activity found to be less sensitive indicator under heavy metals stress than growth inhibition. Similar findings were also documented in *Enteromorpha intestinalis*; which their growth was more impaired with Cu compared to photosynthesis (Lewis *et al.,* 1998). Anyway, the negative effect of these ions could be linked to inhibition of metabolic processes as well as target photosynthetic pathways (Amado Filho *et al.,* 1996, 1997).

Specific growth rate SGR% could be also considered as one of few other factors that affected by heavy metals accumulation in macroalgae. Heavy metals stress provokes a deleterious effect on *U. lactuca* green algae growth and this negative effect was significant ($P < 0.001$) according to applied ion (Fig. 2). Our data showed a higher decline in the growth of algal thali was presented with Cd treatment than the other tested metal ions followed by Pb treatment. While, the lowest value was pronounced with Cu and Zn ions. This observation reflects their capacity to tolerate the latest two ions compared to Cd and Pb.

Previously, Amado Filho *et al.,* (1997) reported the growth and Zn accumulation in six algae species under different levels of Zn. The previous study stated that all six examined seaweeds died 3 days with 5 mg/l Zn. Among the tested algae species, *U. lactuca* died during the first week of 1 mg/l Zn.

Recently, Jiang *et al.* (2013) investigated the physiology and biochemistry changes into *U. prolifera* and *U. linza* exposed to Cd contamination at different concentrations for 7 days. The previous study stated that SGR% was significantly decreased in *U. linza* by 53% and 75% under 0.010 and 0.020 mg/l Cd, while, these values were recorded to be 93% and 39% in *U. prolifera* under the same previous conditions.

The current investigation indicated that all examined toxicants (Pb, Cu, Cd and Zn) had a toxic effect on studied *U. lactuca*. However, the applied concentration for each ion may be found to be over limit that cannot be presented under natural environment conditions. In this respect, Daby (2006), reported that the level of Cu and Zn that could be found along sewage impacted shoreline of Mauritius (Tanzanian coast) was approximately 0.454 mg/l Cu and 0.329 mg/l Zn.

Unal *et al.* (2010) investigated the response of *U. lactuca* to different concentrations of Chromium VI (10, 26, 52 and 260 mg/l) treatment for 2 h. The previous study revealed that, *U. lactuca* was tolerant to Cr VI at low levels (10 and 26 mg/l). Whereas, significant effect was observed with 52 and 260 mg/l. While, algae morphology and photosystem II efficiency were affected under high concentrations (52 and 260 mg/l).

Recently, Chakraborty *et al.* (2014) reported ions accumulation by different algae species that were collected from 6 sites located at the Gulf of Kutch, in India. The previous study showed that the highest Zn concentrations were pronounced in *U. lactuca*.

Loss of chlorophyll was observed with ions adding to the ASW medium. The disappearance of chlorophyll was observed during the first 24 h of treatment. This phenomenon was more pronounced with Pb, Cd and Zn compared to Cu treatment (under their applied concentrations in this experiment). This observation could reflect the sensitivity of algae to Pb, Cd and Zn ions than Cu.

It is worth noting that, sometimes, bioaccumulation of heavy metals varied according to both species specific and metal specific (Daby 2006). Earlier, it has been documented that, the response of the same species differed under heavy metals pollution if it was grown in different environmental conditions (Lee *et al.,* 1998).

Drastic changes in pigments content were also observed after 5 days exposure to heavy metals treatment. This reduction was more pronounced with Pb and Cd treatment compared to the other tested ions. This reflect the highly sensitivity of *U. lactuca* to Pb and Cd ions.

For chlorophyll *a*, significant reduction was more pronounced with Pb (66%) and Cd (64%) treatment. Whereas the lowest one was recorded for Zn (50%) and Cu (53%). While, for Chl *b*, the most reduction was pronounced with Pb (80%) treatment compared to other tested ions.

Moreover, reduction in carotenoids content was also observed for the four tested ions with no significant decrease. Where the highest reduction was found with Cd treatment (22%), while the lowest one was recorded in Zn treatment (20%).

Previously, it has been demonstrated that accumulation of some antioxidants e.g. flavonoids and carotenoids pigments under heavy metals stress, could be considered as one of fewer mechanisms developed by algae and other plants involved in their stress tolerance (Collén *et al.,* 2003). Where, these antioxidants play an important role in elimination and removing of ROS induced after heavy metals exposure (Collén *et al.,* 2003).

In addition, total chlorophyll content was also estimated, our data revealed that the highest reduction in this parameter was recorded for Pb ion (72%) followed by Cd (61%). Whereas, the lowest reduction was pronounced with Zn (54%) followed by Cu (56%) ions.

Jiang *et al.* (2013) reported that no significant decrease in both chlorophyll and carotenoids pigments under 0.010 mg/l Cd than their respective control in both *U. prolifera* and *U. linza* marine algae. While 0.020, 0.040 and 0.080 mg/l Cd, caused reduction in Chl content by 18, 25 and 45% in *U. prolifera*; and it was 16, 20 and 39% for *U. linza.* Whereas, for carotenoids this decline was found to be 16, 29 and 54% in the case of *U. prolifera* and by 13, 16 and 44% below the control for *U. linza,* respectively.

Previously, Gupta and Sarin (2009) reported a decline in chlorophyll and protein content in *Hydrilla verticillata* following their exposure to 0.010 mg/l Cd and 0.005 mg/l Hg for 96 h . While, Caldelas *et al.* (2012) reported a reduction in SPAD values, chlorophyll *a* & *b*, and carotenoids content was recorded in *Iris pseudacours* L. after 5 weeks exposure to 0.649 mg/l of $ZnCl_2$.

Previously, it has been mentioned that heavy metals toxicity could be related to the production of ROS that are known to induce proteotoxicity and changes in protein expression patterns in living organisms (Collén *et al.,* 2003) and cell death (Manoj and Padhy 2013). The previous investigation reported that, living organism exposure's to Zn caused depolarization of mitochondrial membrane on one hand, and mitochondrial dysfunctions especially its electron transport system (ETS) on the other hand.

Collén *et al.* (2003) reported that Cu exposure increase protein expression of defense via oxidative stress. Moreover, other researches showed a decrease in protein content with Cu treatment (Collén *et al.,* 2003; Malea *et al.,* 2006). In this respect, Collén *et al.* (2003) reported that this decline could be found to be 50% after 24 h exposure to Cu in macroalgae *Gracilaria* and other plants *e.g.* rice. The observed decline in Chl *a* & *b* pigmentation after exposure to the mentioned heavy metals, could be related to Mg and Fe deficiency in Chl *a* biosynthetic process. Similar findings were observed with *Gracilaria domingensis* (Rhodophyta) red algae after exposure to cadmium stress for 16 days (dos Santos *et al.,* 2012).

Heavy metals stress induced a decline in osmotic potential compared to the respective control (Fig. 4). It worth noting that, no significant effect was recorded among the four tested ions on this parameter.

This observation showed that with the four heavy metals, treated algae were able to maintain their water status leading to best osmotic regulation and thereby, reduce the heavy metals unfavorable effects on its (Jiang *et al.,* 2013).

From data presented herein, it was noticed that in the case of Pb and Cu treatment, q values were increased as ion concentrations increased from 1.5 to 30 mg/l. While, under Cd and Zn treatment, q values followed an opposite trends. In this regards, max q was observed at 15 mg/l, then it decreased with increased ion concentrations.

The low % ion absorption (R%) and q values proved in this experiment could be explained by the fact, that our experiment has been carried out at pH 7.0. where, this highest pH value caused an increase of the dissociation capacity of functional groups from biosorbent surface. Thereby, these phenomena will be generating an increase of electrostatic interactions as suggested previously by Bulgariu and Bulgariu (2012). Where, the previous study demonstrated that the optimal pH for this type of experiment was pH 5.0 concerning the same algae species exposed to 0.2-3.5 mmol/l of each Pb^{2+}, Cd^{2+} and Co^{2+} ions. Otherwise, the same investigation mentioned that the highest ion absorption was found after 60 min exposure at low temperature.

FT-Raman technique has been employed for investigate and characterize different peaks pattern associated to the functional groups generated by heavy metals absorption of *U. lactuca* green algae. This technique highlighted 4 discriminated peaks ranking from 530.6-3700 cm^{-1} (Fig. 6). Three out 4 peaks were medium, the first one could be linked to CH_2 group (absorption region 1450 cm^{-1}); and the second one of 1700 cm^{-1} could be assigned to C=O group. Whereas, the third peak of 800 cm^{-1} could be assigned to C-O-C group. While the forth of 2950 cm^{-1} one was strong and could be linked to C-H group. From Fig. 6, FT-Raman technique showed that this technique gave similar trends for control and treated plants spectra with no observed differences. Since, among the four tested ions, this technique revealed similar spectra pattern. In spite of the differences occurrences among the four tested ions in term of their nature, solubility, absorption and adsorption, mobility and also their applied concentrations in our case study. Similarly, Ova and Övez (2013) used ART-FTIR spectra to confirm the sorption of Cd^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Fe^{3+} and Cr^{6+} by *Nitzschia closterium*, a marine diatom from waste waters. The latter study showed that the –OH, –NH, –CH stretching vibrations, –C-O stretches, –C–O, –C–C, and –C–OH stretching vibrations, –P–O, –S–O, and aromatic –CH stretching vibrations were the majors functional groups reported in heavy metals absorption into *N. closterium* biomass.

In conclusion, laboratory assay has been done to perform the impact of four heavy metals (Cu, Pb, Zn and Cd ions) against *U. lactuca* algae. *U. lactuca* biomass was more affected by Pb, Cd than Cu while, lowest impact was recorded with Zn treatment compared to their respective control.

It is worth noting that, all examined heavy metals impaired the growth rate and membrane integrity of algae. The highest Chl *a*, *b*; Car, total Chl and osmotic potential values were recorded under Zn treatment; reflecting that this ion could be declared as the lowest toxicant element. Overall, Pb and Cd ions seem to be the most toxicant among tested metals, producing most declines in growth rate % and pigmentation. Overall, the SGR% inhibition was more sensitive indicator for heavy metals toxicity in particularly under Cd treatment.

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