Journal of Stress Physiology & Biochemistry, Vol. 12 No. 2 2016, pp. 62-71 ISSN 1997-0838

ORIGINAL ARTICLE

Lead (Pb) heavy metal impacts in the green *Ulva lactuca* (Chlorophyceae) marine algae

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Received May 5, 2016

Toxicity of different lead (Pb) (0, 2, 4 and 8 mg/L) concentrations in the green *Ulva lactuca* (Chlorophyta) marine algae at physiological level has been investigated 48 h after Pb treatment under laboratory conditions. Thalus algae damages followed Pb treatment as revealed by microscopy test showed that the 4 and 8 mg/L Pb caused morphological changes in cells viability; whereas, no effect observed at the lowest Pb applied concentration (2 mg/L). Data revealed that Pb stress caused reduction in most investigated physiological parameters *i.e.* Pigments content, osmotic potential and membrane stability index values. This decline in osmotic potential was significantly ($p \leq 0.001$) different. Whereas, estimated electric conductivity (EC) values increased significantly ($p \leq 0.001$) as applied Pb concentration increased. The current study allowed somewhat to highlight and better understanding Pb impacts in *U. lactuca* algae. Thereby, the studied algae could be used as a useful bioindicator in Pb polluted ecosystems.

Key words: Ulva lactuca Algae, Physiological parameters, Lead (Pb) toxicity, Heavy metals

According to the U.S. Agency for Toxic Substances and Disease Registry (ATSDR), human activities caused increase in Pb environmental level more than 1.000 fold over the past three centuries. Where, the highest increase occurred between 1950-2000 reflecting the increased use of leaded gasoline worldwide (Pinho and Ladeiro 2012). Pb is considered as not an essential elements for plants; displayed low solubility and availability for plant uptake (Pinho and Ladeiro 2012).

Lead (Pb), mercury (Hg), cadmium (Cd) and tin (Sn) were non-biological essential heavy metals. However, in marine ecosystems, the three metals of Pb, Hg and Cd are of primary concern (Schreiber *et al*., 2002).

Lead as a common environmental pollutant affects living organisms and plant physiological processes in different manner. Their toxicity could be arrived to human through their passage to food chain. The exposure of marine organisms to toxic levels of metal pollutants can cause damage to tissue and growth impair. It has been demonstrated that many marine organisms tend to accumulate heavy metals from the environment (store, remove…) or detoxify many of them. However, their abilities differ between species, making some species more tolerant than others (Kennish 1996).

Several researches reported Pb toxicity as heavy metal and their impact at morphological and physiological levels in micro and macroalgae (Fargasova 1999; Muhaemin 2004; Lamai *et al*., 2005; Ahamad and Shuhanija 2013; Vollanda *et al*., 2014). More recently, Saleh (2015) reported toxicity induced by heavy metals stress (Cu, Zn, Cd and Pb) on *Ulva lactuca* green algae. The latter study showed that Pb ion was the most toxicant element among the tested ions. Thereby, the current study has been conducted to test different Pb concentrations (0, 2, 4 and 8 mg/L) impacts on *U. lactuca* at physiological level in one hand. Moreover, the leakage information regarding response of *U. lactuca* green algae towards Pb ions on the other hands encouraged us to perform this assay.

MATERIALS AND METHODS

Collection of algae samples

Algal samples of *U. lactuca* species were collected along the Syrian coast of the Mediterranean Sea. Sampling was carried out from 34°37′734′′N longitude, 38°29′766′′E latitude at 5 km North Lattakia - Syria. Where, only individual with the similar size was harvested by hand with disposable gloves, biomass was washed with seawater where the algae were collected and then transported within a flask with 5 L seawater.

Algae cultivation and pollution application

U. lactuca species were evaluated under different concentrations of Pb ion under Pb $(NO_3)_2$ forma salt. Upon algae arrival to laboratory, they washed twice 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 divided to a fresh flask with a fresh ASW previously described solution and kept under controlled laboratory conditions [Temperature of 20°C, photoperiod of 12/12 h dark/light and illumination of 3195 Lux (~ 52.7 µmol photons $m^{-2}s^{-1}$)] for 3 days before Pb stress application. The mentioned ASW was considered as a control. Lead stress was applied by adding Pb to achieved 0, 2, 4 and 8 mg/L as final concentration for

each treatment with three replicates/treatment; [(Standard solution (1000 mg/L) from Fisher Scientific –UK, under their nitrate forms)]. Experiment was carried out in flask with 300 mL ASW with or without Pb metal. The same previous described controlled conditions were maintained during the experiment stress application. Algae were harvested 48 h after Pb treatment for physiological study.

Cell viability

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

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 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).

Chl *a* (mg / g FW) = 11.75 A_{662} – 2.35 A_{645}

Chl *b* (mg / g FW) = 18.61 A_{645} – 3.960 A_{662}

Car (mg / g FW) = (1000 A_{470} – 2.270 Chl *a* – 81.4 Chl *b*) / 230

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.

Electric conductivity (EC)

Algal samples (100 mg) were cut into small pieces and taken in test tubes containing 10 mL of double distilled water (ddH_2O); kept at ambient temperature laboratory for 1 h. Algal thalus ions leaching in ddH_2O were measured by a EC meter (HANNA, HI 99301, Hanna Instruments Inc. Woonsocket-RI-USA) instrument.

Membrane stability index (MSI)

This parameter has been estimated by recording the electrical conductivity of algal thalus ions leaching in ddH_2O. Algal samples (100 mg) were taken in test tubes containing 10 mL of ddH_2O in two sets. One set was kept at 40 ˚C for 30 min and another set at 100 ˚C in boiling water bath for 15 min and their respective electrical conductivities, C1 and C2 respectively, were measured by a EC meter (HANNA, HI 99301, Hanna Instruments Inc. Woonsocket-RI-USA) instrument. Membrane stability index was calculated according to the following formula: MSI = [1 – (C1/C2)] × 100.

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 samples of each Pb applied concentrations. Differences between means were tested for significance by Fisher's least significant difference (PLSD) test. Data are expressed as mean of three replicates.

RESULTS AND DISCUSSION

Several reports have demonstrated that uptake of excess metals by living organisms (plants and algae) can generate different metabolic reactions leading consequently to phytotoxic response *e.g.* dwarf growth and chlorosis. The unfavorable effects of heavy metals can caused alteration in plasma membrane permeability thereby, generating ions leakage such K and other solutes (Margaret 1994; Lamai *et al*., 2005). Similarly, Fargasova (1999) reported that the reduction in physiological parameters under Cd, Cu, Zn, Pb and Fe on the green alga *Scenedesmus quadricauda* increased as their concentration increased.

Laboratory experiment has been conducted to study Pb impacts under their applied different concentrations (0, 2, 4 and 8 mg/L) on *U. lactuca* after 48 h exposure.

Light microscopy revealed adverse effects induced on *U. lactuca* after 48 h exposure to Pb ion (Fig. 1). In this respect, 2 mg/L Pb had no effect on algal cells viability. Whereas, when applied Pb concentration increased to 4 mg/L Pb, toxic effect had been noticed reflected in morphological changes; and more noticeable at the highest applied Pb concentration (8 mg/L). Unal *et al.* (2010) reported morphological changes in *U. lactuca* exposed to different Chromium VI concentrations (38.84, 97.09, 194.19 and 970.95 mg/L K_2CrO_4) for 2 h. Similar findings were also recently reported in response of the same algae species to Cu, Zn, Cd and Pb heavy metals for 5 days (Saleh 2015). Whereas, Vollanda *et al*. (2014) reported that Pb ions cause shape changes and cells death combined with no structural alteration in cytoplasm under light microscopy in unicellular *Micrasterias* green algae after 21 days exposure.

Decline in most investigated physiological parameters was recorded due to Pb stress. As presented in Fig. 2, pigments content (Chl *a*, Chl *b*, Car and total Chl) decreased as Pb applied concentrations increased.

Our data showed that Chl *a* values decreased by 1.32, 22.7 and 40.4% at 2, 4 and 8 mg/L Pb. Whereas, this reduction was recorded to be 15.8, 13.4 and 17.7% for Chl *b* under the above mentioned Pb concentrations. While, Car content decreased in similar trend (23.4 – 23.7% at 2 – 8 mg/L Pb) regardless applied Pb concentration. This observation could be serving as a protective mechanism developed by studied algae via unfavorable effects of Pb. Where, Car play an important role as antioxidant non enzymatic systems in Pb detoxification or in alleviation the Pb toxicity by escape induced reactive oxygen species (ROS) followed Pb stress. Moreover, total Chl decrease from 6.2 to 32.7% as Pb applied concentration increased from 2 to 8 mg/L.

To earlier, Pahlsson (1989) reported that both of Cd and Pb inhibited plants chlorophyll biosynthesis, leading to the lowered chlorophyll contents.

Previously, Muhaemin (2004) reported toxicity of different Pb (0.025, 0.050, 0.100 and 0.150 mg/L) concentrations against two green unicellular *Chlorella* and *Dunaliella* algae, for 6 days. The previous study showed that *Dunaliella* was most tolerant to Pb than *Chlorella* algae. Where algae with smallest size showed highest sensitivity to Pb. Whereas, Lamai *et al*. (2005) reported toxicity of different Pb (5, 10, 20, 40 and 80 mg/L) and Cd (0.5, 1, 2, 4 and 8 mg/L) concentrations during 2, 4, 6 and 8 days exposure in the green algae *Cladophora fracta*. The latter investigation showed reduction in chloroplasts number with several damages followed Pb and Cd

treatment. Where, the chloroplast is the organelle most affected by metal contamination. Moreover, the previous study revealed significant decline in relative growth and total chlorophyll values as exposure time and ions applied concentrations were increased. However this inhibition was not significant after 2 days exposure to both Pb and Cd ions, compared to their respective control. In this respect, the lowest total Chl content was recorded to be 1 mg/g with 80 mg/L of Pb and 1.1 mg/g with 8 mg/L of Cd after 8 days exposure. It worth noting that, in our case study, applied Pb concentrations were much lower (10-folds less) than those used in Lamai *et al*. (2005) investigation.

Moreover, Szivak *et al*. (2009) reported that both the Pb and Cd ions were highly phytotoxic to living organisms leading to growth impair and even death. Whereas, Carfagna *et al*. (2013) investigated physiological response of *Chlorella sorokiniana* microalgae exposed to 50.33 mg/L Cd and 81.32 mg/L Pb ions for 2 h and 24 h of treatment. The latter study showed decease of algae growth rate of 2.2/day and 1.65/day for Pb and Cd, respectively. Moreover, heavy metals stress caused significant reduction in photosynthetic rate by 59% and 77% after 2 h and 24 h Pb exposure, respectively. Whereas, this inhibition was found to be 77% and 14% after 2 h and 24 h Cd, respectively. Otherwise, Pb treatment caused drop in Chl *a* and total Chl by 75.5% and 59.8%, respectively after 24 h. Whereas with Cd treatment, this reduction was recorded to be 97.8% and 67.9%, respectively. Indeed, Ahamad and Shuhanija (2013) studied Cu, Pb and Hg (2 mg/L) toxicity after 24 h exposure to the mentioned metals in *Gracilaria manilaensis* red algae at physiological and biochemical levels. The latter investigation revealed that Chl *a* florescence significantly decreased by approximately 34% and 22% with Cu and Pb treatment, respectively. Whereas, Hg was the most toxicant ion followed by Cu and Pb. It has been documented that Pb can decrease photosynthesis rate through distorting chloroplast ultrastructure, decline Chl synthesis, obstructing electron transport and impair activities of Calvin cycle enzymes (Sengar *et al*., 2008).

Recently, Saleh (2015) investigated physiological alterations generated 5 days after exposure heavy metal pollutants (Cu, Pb, Zn and Cd ions) on *U. lactuca.* The latter study showed some morphological changes in algal thali with heavy metals stress. Moreover, heavy metals caused a decline in studied physiological parameters (Chl *a*, Chl *b*, Car and total Chl). This reduction was more noticeable with Pb ion compared to the other tested metals. In this respect, Pb caused decline in Chl *a*, Chl *b*, Car, total Chl by 66%, 80%, 20% and 72%, respectively.

As for osmotic potential as a direct response of living organisms to an abiotic stress, has been estimated (Fig. 3). Data revealed that Pb treatment caused a significant decrease in this parameter ($p \leq 0.001$); where their decline increased from 13-38% as Pb applied concentration increased from 2 to 8 mg/L. Saleh (2015) reported that Pb ions heavy metal 5 days after exposure on *U. lactuca* reduced potential osmotic by 68%.

Agal thalus ions leaching towards Pb treatment has been measured compared to their respective control (Fig. 4). Significant increase in EC value ($p \leq 0.001$) ranged from 0-20% when Pb applied concentration increased from 2-8 mg/L. Other investigation carried out on the same algae species treated with different chromium (VI)

(38.82, 97.09, 194.19 and 970.95 mg/L K_2CrO_4) concentrations for 2 h, revealed that EC values increased as Cr concentrations increased (Unal *et al*., 2010).

Membrane stability index values were also calculated to highlight different Pb concentration impacts on *U. lactuca* (Fig. 5). Results indicated that Pb stress caused a progressive decline in this parameter as Pb applied concentration increased. Similar observations have been recorded in other algae species under salinity stress (Al-Abssy and Al-Hakimi 2010). The latter study reported that salinity (23377 mg/L of NaCl) caused decline in MSI *Chlorella fusca* microalgae compared to their respective control.

Vollanda *et al*. (2014) reported the effect of additive of some antioxidants and microelements such as Zn, Ca and Fe to alleviate the negative effects of Cd, Pb and Cr heavy metals on unicellular *Micrasterias* green algae. The latter study showed that the additive agents diminish the negative effects of heavy metals stress with different manners. Moreover, Pb ions showed algal shape changes and cells death. Whereas, light microscopy showed no structural alteration in cytoplasm; suggesting that this element dose not enter the cell. Moreover, 1.66 mg/L Pb caused decline in cell vitality by 76.37% after 21 days exposure, compare to their respective control.

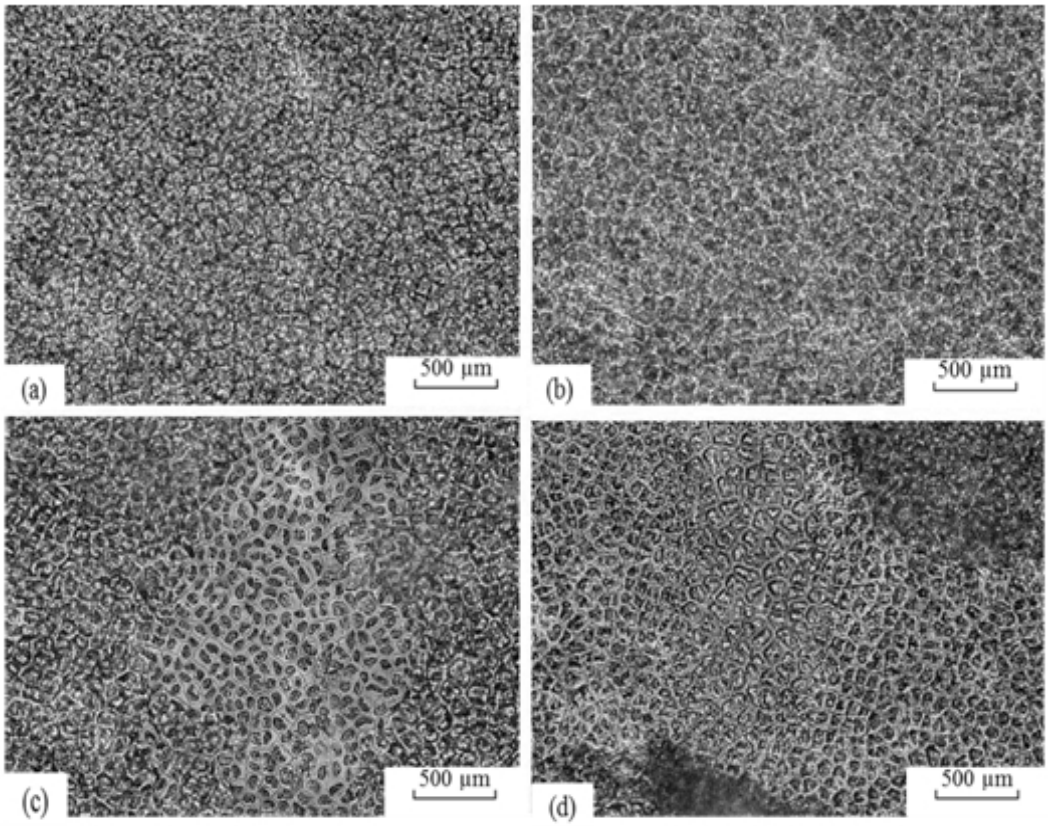

Figure 1. *U. lactuca* morphological changes induced after 48 h exposure to different Pb concentrations, as revealed by microscopy test. (a): Control; (b): 2 mg/L Pb; (c): 4 mg/L Pb and (d): 8 mg/L Pb.

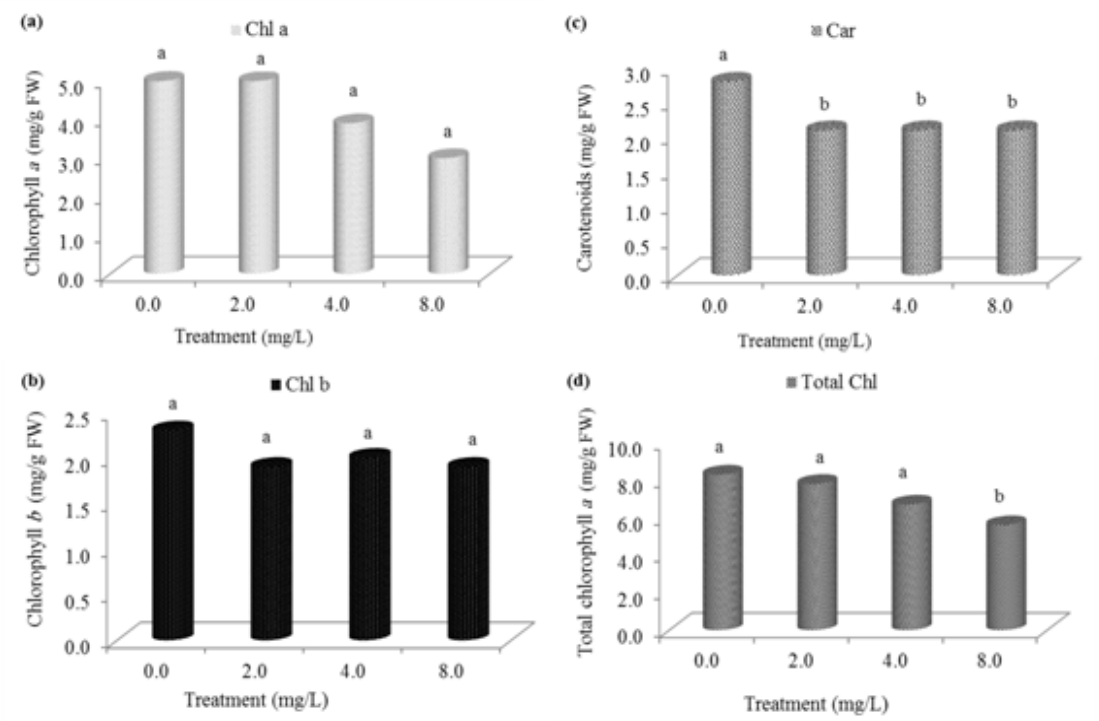

Figure 2. Pigments content of *U. lactuca* algae under control and different Pb concentrations, 48 h after exposure to Pb stress. (a): Chlorophyll a; (b): Chlorophyll b; (c): Carotenoids and (d): Total chlorophyll (mg/g Fresh weight) (n=3).

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: 0.917; $LSD_{0.05}$ Chl b / treatment: 1.169; $LSD_{0.05}$ Car / treatment: 0.394 and $LSD_{0.05}$ Total Chl / Treatment: 1.721.

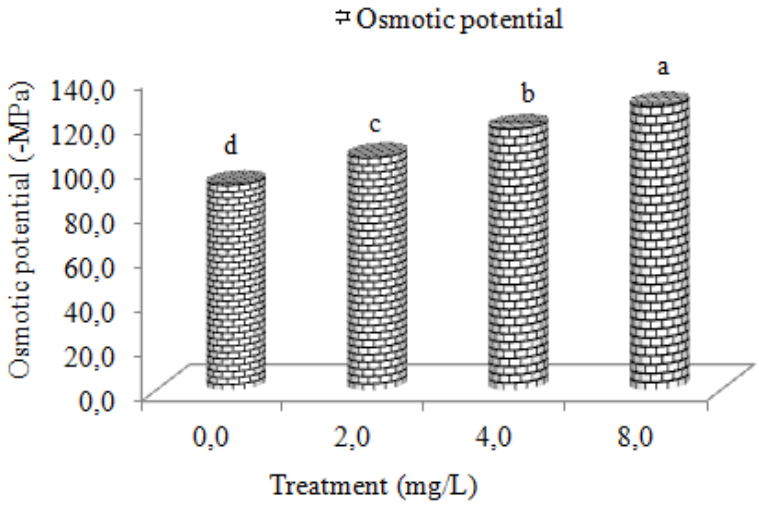

Figure 3. Osmotic potential (–MPa) of *U. lactuca* under control and different Pb concentrations, 48 h after exposure to Pb stress (n = 3).

Notes. Figures sharing the same lowercase letter are not significantly different at p = 0.05 probability by Fisher's PLSD test. $LSD_{0.05}$ osmotic potential / treatment: 5.489.

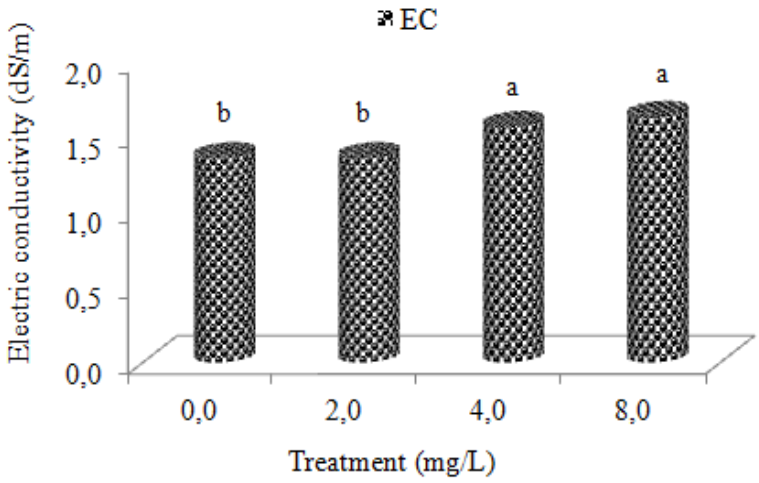

Figure 4. Electric conductivity (EC) of *U. lactuca* under control and different Pb concentrations, 48 h after exposure to Pb stress (n = 3).

Notes. Figures sharing the same lowercase letter are not significantly different at p = 0.05 probability by Fisher's PLSD test. $LSD_{0.05}$ EC / treatment: 0.081.

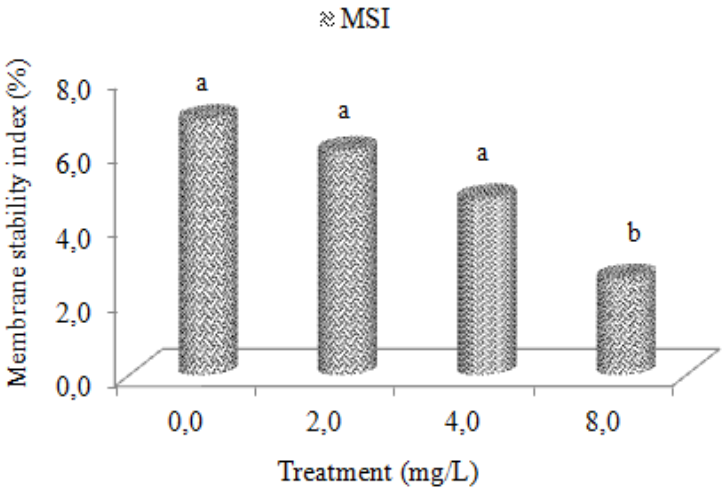

Figure 5. Membrane stability index (MSI) (%) of *U. lactuca* under control and different Pb concentrations, 48 h after exposure to Pb stress (n = 3).

Notes. Figures sharing the same lowercase letter are not significantly different at p = 0.05 probability by Fisher's PLSD test. $LSD_{0.05}$ MSI / treatment: 2.543.

CONCLUSION

Data presented herein demonstrated for the first time light microscopy alterations combined with different physiological parameters induced by different Pb concentrations on *U. lactuca*. The similar drop on Car content (23.4 – 23.7% at 2 – 8 mg/L Pb) regardless applied Pb concentrations; could be suggested that the studied algae able to develop adaptation mechanism via

Pb stress. Further researches on DNA damages induced by Pb stress could help to illustrate a repair mechanism involved in Pb stress tolerance.

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