ANALYTICAL METHODS EMPLOYMENT FOR HEAVY METALS POLLUTION INVESTIGATION IN *ULVA LACTUCA* (CHLOROPHYCEAE) SEAWEED

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(Received on Date: 2nd May 2017 Date of Acceptance: 27th July 2017)

ABSTRACT

Effect of 4 metal pollutants (Pb, Cu, Cd and Zn) 5 days exposure in *Ulva lactuca* seaweed has been evaluated to elucidate their deleterious impacts into the mentioned seaweed. Heavy metals stress application adversely affected sea weed elemental (C, H and N) content. In this respect, the highest carbon content was observed under Zn ions, whereas, the lowest one was recorded in the response to Pb ions. Moreover, Fourier transform infrared (FTIR) spectroscopy as an analytical measurement has been employed to highlight the probable binding sites of metal pollutants in *U. lactuca* seaweed. In this respect, FTIR spectra revealed O-H, N–H, –C–O, P-O-C, S-O, C-C, -C-C, –C-OH, C-H and C-I groups distinguished peaks as function groups that could be involved in heavy metals detoxification. Based on our data, FTIR technique proves to be a potential tool for monitoring structural changes and probable binding sites induced by metal pollutants in the marine environment.

Keywords: Fourier transform infrared spectroscopy (FIIR), elemental analysis, heavy metal pollutants, *Ulva lactuca*

INTRODUCTION

According to the U.S. Environmental Protection Agency (EPA), of heavy metals, lead, cadmium and mercury are the three most environmental pollutants (ATSDR, 1999). Heavy metals stress stimulates induction of some components that act as protective mechanisms via unfavorable impacts of different pollutants. In this respect, plant, algae and fungi accumulate molecules such as metallothionein (MT), glutathione(GSH), phytochelatins(PCs), and plastocyanin (Cu^{2+}Pc)(Gekeler *et al*., 1988;Damodaran *et al*., 2013). Gekeler *et al*. (1988) reported that algae and higher plants tend to synthesis phytochelatin complexes as an osmolyes to protect themselves against toxicity of metal pollutants (Cd, Pb, Zn, Ag, Cu and Hg).

Environmental pollution effect could be measured quantitatively or/and qualitatively. Exposure of living organisms such as sea weeds to chemical pollutants causes adverse effects. Heavy metals stress impacts on seaweeds have been intensively investigated based on physiological and biochemical levels. Moreover, limited research focused on molecular mechanisms involved in heavy metals response in seaweeds (Saleh 2015a; b; Saleh 2016). Plants respond to unfavorable conditions by developing different mechanisms at physiological, biochemical and molecular levels. The maintenance of carbon (C) and nitrogen (N) assimilation forms as an essential factor playing an important role as a protective mechanism in stress response. Where, the interaction between C and N metabolism forms a potential factor for different stages development of plant (Nunes-Nesi *et al*., 2010). Coruzzi and Zhou (2001) reported that C and N activate signaling pathway involved in transporter activities and enzymes regulation process which control C and N fluxes leading consequently to enhancement of plant tolerance to environmental stresses. Seaweeds with their useful characteristics *e.g*. their potentiality to accumulate high amount of metal pollutants in their tissues, their rapid growth rate; their fast interaction with the environment where they grow, their morphological structure simplicity and their abundance at sea coast, makes them a good biomass choice for elimination of different heavy metals at a low cost. Also, they make a good candidate for investigation of heavy metals pollution in ecosystems. However, very limited reports investigated cellular mechanisms involved in heavy metals detoxification and the subsequent increase in their tolerance to metals stress. Many analytical tools could be employed to study mechanisms of metals de toxification.FT-IR as an analytical method proved advantageous compared to other techniques. Their importance stems from their simplicity of sampling, non-destructive method, rich qualitative and quantitative information content and very small samples needed. Immense interest in their application appeared in recent years as a simple and rapid tool among other analytical methods. FTIR was applied for the first time by Morikawa and Senda (1974) for studying divalent cation binding to cell walls through pectic polysaccharides and carboxyl groups on algal cell wall (Morikawa and Senda 1974; Morikawa *et al*., 1974).

Several researches reported different founctional groups involved in reaction of seaweeds to metal exposure using FT-IR technique. *e.g.*in *Padina tetrastromatica* (D'Souza *et al*.,2008), *Kappaphycus alvarezii* seaweed waste (Lee *et al*., 2011),*Acanthophora spicifera* seaweed (Tamilselvan *et al*., 2011), *Caulerpa racemosa* green seaweed (Dekhil *et al*., 2011) and in *Nitzschia closterium*, a marine diatom (Ova and Ovez 2013).

To our knowledge, no report till now has described heavy metals impact on the green *U. lactuca* seaweed using FTIR analysis. Thereby, the current investigation focused on elemental analysis (N, C and H content) and FTIR assays as an analytical methods to quantify 4 metal pollutants impact in *U. lactuca* seaweed.

MATERIALS AND METHODS

Collection of seaweed

U. lactuca seaweed with similar sizes were collected from the Syrian coast of the Mediterranean Sea (35°33′790″N longitude, 35°43′996″E latitude)4 km North Lattakia - Syria.Sea weed samples were washed with seawater, then transported in a flask containing 5 L fresh seawater.

Pollutant application

Seaweed samples were washed twice with autoclaved artificial seawater ASW medium as described by Unal *et al*. (2010). Samples were then divided and transformed to a fresh flask with fresh ASW solution and kept under controlled 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 metal stress application.

Some samples were kept in flask with fresh ASW as control. Whereas, metals pollution was carried out by adding 18.2 mg/L of Pb, 5.8 mg/L of Cu, 10.5 mg/L of Cd and 9,9 mg/L of Zn [(Standard solution (1000 mg/L) from Fisher Scientific –UK, in their nitrate forms)] for each treatment with five replicates/ treatment. Laboratory experiment was performed in flasks with 300 mL ASW with or without heavy metals. The same previously described controlled conditions were maintained during the experiment. Seaweed was harvested after five days exposure to metals.

Elemental analysis

Seaweed tissues were oven-dried at 60°C overnight, crushed in QIAGEN Retsch model Tissue Lyser II (USA) and stored at room temperature. Elemental analysis was performed by EURO EA (Elemental Analyzer L1-K4) apparatus.

Fourier transform infrared spectroscopy (FI-IR) technique

A hundred mg of seaweed tissues for each treatment were ground and 5 ml of 80%

acetone were added; then samples were placed at 4°C for 24 h. Tubes were centrifuged at 1400 g/ 2 min. Then, filtration of extracts was carried out using Whatman filter papers as reported by Saleh (2015a). These filtrated extracts were used as template for FT-IR measurement using NXR FT-IR (Thermo, USA) instruments.

Statistical analysis

Data were statistically analyzed using Statview 4.5 (Abacus 1996) statistical package at the 5% significance level (*P* = 0.05). Analysis of variance (ANOVA) for the determination of differences between means of untreated and treated seaweed has been performed. Differences between means were tested for significance by Fisher's least significant difference (PLSD) test. Data are expressed as mean of five replicates.

RESULTS AND DISCUSSION

Quantification test of essential compounds including carbon, hydrogen and nitrogen has been performed. Elemental analysis showed that heavy metals stress induced an increase in N, C and H compared to the control (Table 1).

Table 1. Elemental analysis of N, C and H content into *U. lactuca* seaweed, 5 days after heavy metal stress

Elements	Control	Pb	Cu	Cd	Zn
N	1.8±0.1b	2.1±0.3b	1.9±0.8b	2.5±0.3a	2.5±0.4a
C	26.7±1.9c	31.9±1.1b	37.3±1.1a	37.1±1.4a	39.4±1.6a
H	5.9±1.6a	6.6±1.5a	7.2±1.3a	5.7±2.3a	6.8±1.3a

LSD5% N=0.811, LSD5% C=2.574 and LSD5% H=2.985

The most observed increase was pronounced in carbon%. In this respect, this value increased by 20, 40, 39 and 47% for Pb, Cu, Cd and Zn ions, respectively over the control. From this observation it could be assumed that seaweed tend to augment their content of organic matter as a developed protective mechanisms against the deleterious effect provoked by metal pollution. Variance analysis of the heavy metals stress on N, C and H content into *U. lactuca* seaweed, revealed that heavy metal stress has significantly affected C content according to each treatment **(**Table 2**).**

Table 2. Variance analysis of the heavy metals stress on N, C and H content into *U. lactuca* seaweed

Elements	DF	Sum of Squares	Mean square	F-Value	P-Value
N	4	1.331	0.333	1.674	0.2316
C	4	318.355	79.589	39.756	< 0.0001
H	4	4.841	1.21	0.449	0.7709

A possible explanation to these observations could be that seaweed tends to synthesize higher quantities of organic compounds (N, H, and C) under metal stress as compared to the control to avoid oxidative stress and ROS production following exposure to pollutants.

Reguera *et al*. (2013) reported drought stress impacts on C and N metabolism in rice leaves of wild-type and transgenic plants. The previous investigation showed a good correlation between sustained carbon assimilation in the transgenic plants and nitrate content enhancement, higher nitrate reductase activity, and sustained ammonium contents, referring that cytokinin synthesis induced by stress in transgenic plants exhibited a role in nitrate acquisition maintenance. Indeed, photosynthesis activity was maintained under drought stress. The previous investigation revealed that induced cytokinin synthesis by drought stress in transgenic plants leads to improve rice tolerance to water deficit.

In the current study, impact of Pb, Cu, Cd and Zn metals on C, N and H content was investigated in *U. lactuca* seaweed. Data revealed that applied chemical pollutants significantly increased C content ($P < 0.001$). In this respect, C value recorded to be 26.7, 31.9, 37.3, 37.1 and 39.4 under control, Pb, Cu, Cd and Zn ions, respectively. Whereas, non significant increase in N content was recorded under heavy metals treatment. While, H content remains stable in control and metals treatment regardless of applied ions. Moreover, C/N ratio was also estimated under chemical stress compared to the control. In this respect, data revealed that metal stress increase this ratio. Where, this value was recorded to be 14.8, 15.2, 19.6, 14.8 and 15.8 for control, Pb, Cu, Cd and Zn ions, respectively.

Recently, Yadav *et al*. (2015) reported Cr, Cd, Pb and Zn pollutants effect on C and N in Indian *Avicennia marina* in comparative study between polluted and non-polluted regions. The previous investigation revealed that C/N average ratios were recorded to be 80.1, 105.8, and 52.4 in three polluted regions. Whereas, this value recorded to be 26.4 in non-polluted one. The previous study suggested that the above metals caused a decline in C amount in plants leading to alteration C/N ratio.

However, other investigation carried on the same species under the same pollution conditions, revealed that Pb ion was the most toxicant ion against studied *U. lactuca* as it showed the highest decline in physiological parameters [specific growth rate (SGR%), chlorophyll (Chl *a*&*b*), total chlorophyll and carotenoids (Car) content)] (Saleh 2015a). The previous investigation revealed that an inverse tendency was recorded under Zn and Cu treatment; with the seaweed showing the lowest sensitivity to the latter ions.

Moreover, other study focused on measurement of DNA changes generated by the mentioned ions in the same species based on random amplified microsatellite polymorphism (RAMP) technique (Saleh 2015b). The previous investigation stated that RAMP marker successfully highlighted

DNA change patterns induced by metals stress. Indeed, the same study may suggest that similar tendency at genomic DNA alterations has been recorded with Cd and Zn ions. Similar finding was also observed with Pb and Cu ions. The previous study assumed that *U. lactuca* seaweed could be used as potential bio indicator to investigate heavy metals pollution. This enhanced growth was also confirmed from the quantities of essential elements *e.g.* the elemental analyses showed that the carbon content was significantly higher under Zn treatment; whereas, the lowest values were recorded in the case of Pb ions. These findings strongly support and confirm recent data obtained by Saleh (2015a).

However, FT-IR spectrum in the mid-infrared region (500–4000 cm^{-1}) has been employed for characterization of different functional groups in *U. lactuca* seaweed after 5 days exposure to Pb, Cu, Cd and Zn ions (Figure 1). Variation in spectral features of the IR bands of *U. lactuca* (control and treated) suggests that metals pollution bind to the induced different specific bands (Figure 1).

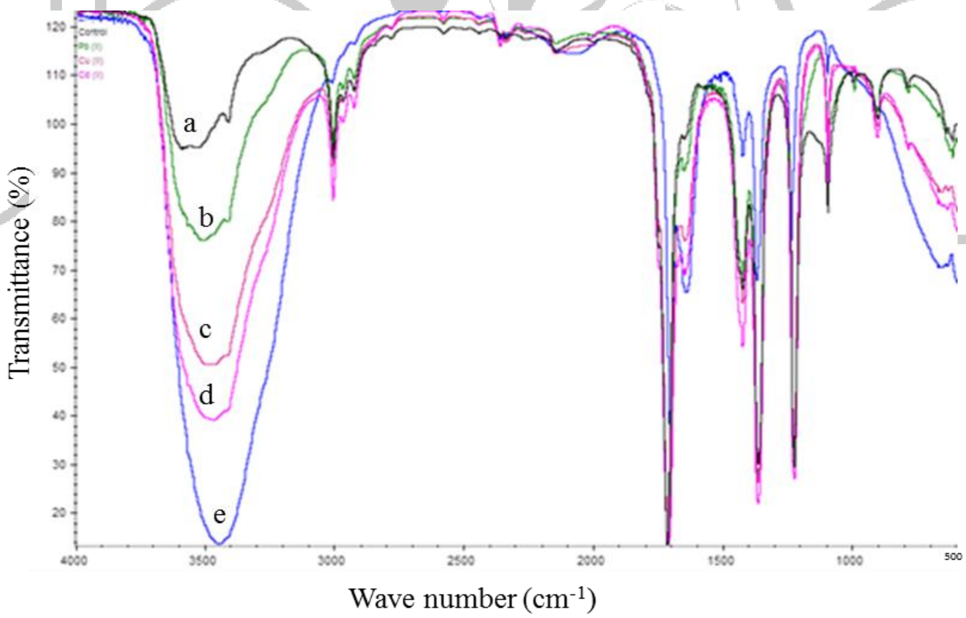

Figure 1. FT-IR spectra of untreated (a), Pb-treated (b), Cu-treated (c), Cd-treated (d) and Zn-treated (e) *U. lactuca* seaweed, after 5 days exposure.

FT-IR technique highlighted 9 discriminate peaks ranged from 530.6 to 3588.8 cm^{-1}. The peak around 3588.8 cm^{-1} could be linked to O-H group (absorption region 3640-3530 and 3620-3540 cm^{-1}). The peak around 3450 cm^{-1} could be related to N–H amino compound (Aromatic secondary amine, NH stretch). Whereas, the peaks of 1363.5, 1421.6and 1715.4 cm^{-1} indicative of –C–O group. While, the peak of 1222.9 cm^{-1} could be related to P-O-C stretch group. Indeed, the peak of 1363.5 cm^{-1} could be attributed to S-O group. Moreover, C-C stretch (skeletal vibrations) could be observed in the peak absorption region of 1350-1000 cm^{-1}. In addition, -C-O; -C-C and –C-OH stretch vibrations could be presented at the peak absorption region of 1300-1000 cm^{-1}. The peak around 903.4 cm^{-1} could be linked to C-H group, while the peak of 530.6 cm^{-1} could be assigned to C-I stretch at the peak absorption region of 600-500 cm^{-1}.

Recently, Saleh (2015a) applied FT-Raman technique for screening different peaks associated to the functional groups yielded in *U. lactuca* seaweed following heavy metals absorption. The previous investigation revealed 4 distinguished peaks ranged from 530.6 to 3700 cm^{-1}. 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.

D'Souza *et al*. (2008) applied FT-IR to screening various peaks involved in *Padina tetrastromatica* (Hauck) exposed to Cd stress (50 ppm) for 3 weeks. The previous investigation revealed that Cd treatment induced hydroxyl, amino, carbonyl and phosphoryl function groups. In this respect, a band at 3666 cm^{-1} in control *P. tetrastromatica,* while a band at 3560 cm^{-1} in Cd-treated *P. tetrastromatica* due to non bonded and bonded O-H, respectively were recorded. Indeed, two bands of 3500 and 3450 cm^{-1} in control sample corresponding to N-H stretching vibrations and a band of 1577 cm^{-1} corresponding to N-H bending vibrations appeared. Whereas, in treated algae an intense band at 3350 cm^{-1} corresponding to N-H stretching vibrations and 1571 cm^{-1} due to bending vibrations were observed. Moreover, a band of 1764 cm^{-1} due to ester carbonyl group appeared into algae control sample; whereas, Cd stress induced a band of 1760 cm^{-1}. It was noticed that, decline in band intensity of 1710 cm^{-1} in control sample followed Cd stress referring that carbonyl of COOH to be included in metal chelating. Indeed, a band of 1224 and 1220 cm^{-1} for control and treated algae, respectively referred to the phosphoryl group employment in metals binding.

Whereas, Lee *et al*. (2011) used FTIR analysis to evaluate functional groups involved in Cr, Ni, Cd, Cu and Zn metals absorption in *Kappaphycus alvarezii* seaweed waste. The previous investigation showed that the functional groups related to metals absorption were 1050 cm^{-1} (–C–

O groups), 1646 and 1240 cm^{-1}(–COOH groups), 2350 cm^{-1} (–C≡N groups), 2930 and 1385 cm^{-1} (–CH3 groups) and 3420 cm^{-1} (–OH or –NH groups).

Moreover, Ova and Ovez (2013) applied ART-FTIR spectra to study the sorption of Cd^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Fe^{3+} and Cr^{6+} into *Nitzschia closterium*, a marine diatom from waste waters. The previous investigation showed that the majors functional groups revealed in the above metals absorption into *N. closterium* biomass were –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. Whereas, Damodaran *et al*. (2013) applied FT-IR technique to characterize the different groups involved in Cr(VI) stress into *Galerina vittiformis* Mushrooms. The previous study showed that Cr(VI) induced oxalic acid (1658 and 1253 cm^{-1}) and thiol group (2550 cm^{-1}) as function groups.

Tamil selvan *et al*. (2011) investigated detectable functional groups as a result of *Acanthophora spicifera* algae exposure to Cr, Pb, Cd and Hg heavy metals stress using FT-IR. The previous study showed that carboxyl (–COOH), hydroxyl (–CHOH) and amine (–NH2) groups were involved in detoxification of the applied heavy metals. Whereas, Dekhil *et al*. (2011) reported functional groups involved in Pb(II) and Cd(II) ions sorption in the *Caulerpa racemosa* green seaweed using FT-IR. The previous investigation revealed O–H bending, N–H stretching, C–N stretching, C–O and S=O stretching as functional groups detected in response of algae to the tested ions.

Whereas, Durve and Chandra (2014) investigated *Pseudomonas aeruginosa* bacteria response to metals stress (Cd, Hg, Pb and As) using FT-IR technique. The previous investigation revealed O-H stretching, S-O and N-H were detected as a response of bacteria to the 4 applied metals; where as, C-O-C, C-O and C-O-P were detected in bacteria as a response to Cd and Pb.

More recently, other investigation (Saleh 2016) however revealed comparative study between *U. lactuca* (Chlorophyta) green and *Padina pavonica* (Phaeophyta) brown seaweeds exposed to different cadmium (Cd) (0, 2.5, 5 and 10 mg/L) concentrations for 4 days. The previous investigation showed that *P. pavonica* was more tolerant to Cd stress compared to *U. lactuca* based on different physiological parameters [specific growth rate (SGR%), pigmentation (Chlorophyll a & b, total chlorophyll and total carotenoids), electric conductivity (EC) and osmotic potential].

In conclusion, impact of four heavy metals (Pb, Cu, Cd and Zn) after 5 days exposure in *U. lactuca* seaweed was screened based on C, N and H% content and FTIR technique. Elemental analysis showed that algae treated with Zn displayed the highest C%, whereas, samples treated with Pb displayed the lowest one. FTIR spectra successfully identified O-H, N–H, –C–O, P-O-C, S-O, C-C, -C-C, –C-OH, C-H and C-I as functional groups that could be involved in heavy metals detoxification.

ACKNOWLEDGEMENTS.

I thank Professor Othman Ibrahim (director general of AECS) and Professor MirAli Nizar (head of Molecular Biology and Biotechnology Department in AECS) for their support, and also the Plant Biotechnology Group for technical assistance.

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