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ScienceDirect

Procedia Environmental Sciences 30 (2015) 222 – 227

Procedia Environmental Sciences

International Conference on Environmental Forensics 2015 (iENFORCE2015)

Impact of mercury (II) nitrate on physiological and biochemical characteristics of selected marine algae of different classes

Luqman Abu Bakar[a], Nakisah Mat Amin[a], Hazlina Ahamad Zakeri[a]*

[a]*School of Fundamental Science, Universiti Malaysia Terengganu (UMT), 21030 Kuala Terengganu, Terengganu, Malaysia*

Abstract

Short-term physiological and biochemical responses induced by mercury(II) nitrate ($Hg(NO_3)_2$) in three marine macroalgae, *Gracilaria salicornia* (Rhodophyceae), *Sargassum* sp. (Phaeophyceae) and *Ulva reticulata* (Chlorophyceae) were studied. All algae were good accumulators of Hg. However, this characteristic significantly decreases in *G. salicornia* and significantly increases in *U. reticulata* as concentration of Hg increases. Algal F_V/F_M, chlorophyll (chl) a content and relative growth showed a significant reduction in the presence of Hg. Hg impact was prominent in F_V/F_M than the other parameters while a small impact was shown by the relative growth. Hg induced synthesis of new amino acids and inhibit the production of some. The profile even changed when the concentration of $Hg(NO_3)_2$ increases.

Peer-review under responsibility of organizing committee of Environmental Forensics Research Centre, Faculty of Environmental Studies, Universiti Putra Malaysia.

Keywords: heavy metals; marine alga; maximal quantum yield; chl a content; amino acid content

1. Introduction

Mercury (Hg) is recognized globally as an important pollutant. Hg and its compounds are persistent, bioaccumulative and toxic. Hg contamination can represent a serious threat to humans and ecosystems. In aquatic ecosystem, inorganic Hg is the most common form of Hg released in the aquatic environment by industries.

* Corresponding author. Tel.: +609-6683357; fax: +609-6683608
E-mail address: hazlina@umt.edu.my

Peer-review under responsibility of organizing committee of Environmental Forensics Research Centre, Faculty of Environmental Studies, Universiti Putra Malaysia.
doi:10.1016/j.proenv.2015.10.040

Biochemical mechanisms involved in cellular detoxification are particularly significant in understanding the harmful effects of Hg or other environmental pollutants. In addition, they can also be useful biomarkers of exposure to aquatic pollutants. Photosynthesis, an important metabolic process for the autotrophs has been known to be very sensitive to heavy metals. Hg is able to alter the photosynthetic machinery including the chloroplastic photosystem I (PSI) reaction center subunit II, the oxygen-evolving protein and the chloroplastic ATP synthase β-subunit [1]. In this study, chlorophyll (chl) fluorescence analysis was used as a useful physiological tool to assess early stages of change in photosynthetic performance of algae in response to heavy metal pollution [2]. This method has been shown to be rapid, non-invasive, and reliable for assessing photosynthetic performance in a changing environment [3]. Among the parameters of chl fluorescence, the maximal quantum yield or F_V/F_M has been widely used and is directly proportional to the quantum efficiency of PSII photochemistry [4]. The toxicity of metals and their compounds, however, largely depends on their bioavailability, that is, the mechanisms of uptake through cell membranes, intracellular distribution, and binding to cellular macromolecules [5]. High levels of Hg in the form of Hg^{2+}, have strong phytotoxic effects and when present in toxic concentrations can induce visible injuries and physiological disorders in plant cells triggering the production of reactive oxygen species (ROS) leading to cellular disruption [6]. Hg and other metals can become a problem because they cannot be easily degraded or destroyed. Nevertheless, they can be removed from the contaminated water bodies. Since most conventional methods are neither effective nor economical, new separation methods are required to reduce heavy metal concentrations to environmentally acceptable levels at affordable cost. Bioremoval, the use of biological systems for the removal of metal ions from polluted waters, has thus, the potential to contribute to the achievement of this goal [6]. Bioremoval can be achieved by biosorption or bioaccumulation processes [7]. Macroalgae play a major role in marine ecosystems. As the first organism in marine food chains, they provide nutrients and energy for animals as well as provide shelter and habitat for scores of coastal animals. Macroalgae require inorganic nutrients for growth. The fast-growth rate of some species of macroalgae can account for rapid nutrient removal from marine waters. Most of them are able to immobilize the metals to make them less toxic [8]. In addition, they have the ability to adsorb and metabolize trace metals due to their large surface:volume ratios, the presence of high-affinity, metal-binding groups on their cell surfaces, and efficient metal uptake and storage systems [9]. These characteristics make them suitable as bioremoval material.

The aim of this study is to determine the physiological and biochemical responses of three marine macroalgae, *Gracilaria salicornia*, *Sargassum* sp. and *Ulva reticulata* against Hg in terms of Hg accumulation in the tissues, F_v/F_M, chl a content, relative growth and amino acids composition. This study is a preliminary study on the potential use of these algae as a bioremoval as well as bioindicator of the Hg-polluted waters.

2. Materials and methods

The red alga, *Gracilaria salicornia*, brown alga, *Sargassum* sp. and green alga, *Ulva reticulata* were collected from the coastal of Port Dickson, Negeri Sembilan, Malaysia and further cultivated at the Marine Hatchery, Universiti Malaysia Terengganu in an open tank system. For the metals treatment, ~5 g of the alga was treated with 3.1 μM and 6.2 μM of mercury(II) nitrate ($Hg(NO_3)_2$) for 8 hrs in aerated beakers containing filtered seawater under white light. The conditions used for control was similar as above but with no additional metals (i.e. untreated alga). Each experiment was done in triplicates. Prior to treatment, algal fresh weight (FW) was measured. This FW will be compared with that of the FW measured after the treatment. Relative growth was then calculated as FW after treatment divided by FW before treatment. The amount of Hg in the algal tissues (~30 mg) was determined using the MA-3000 Mercury Analyzer (Nippon Instruments, North America). The unit of Hg measured was in ppb which was then converted into mg/kg algal dry weight.

Chl a fluorescence was measured with a handheld fluorometer, AquaPen-P AP-P 100 (Photon Systems Instruments, Czech Republic). At the start of the measurement, a short, red, actinic pulse (~3000 μmol m^{-2} s^{-1} at 655 nm) was prompted for 5 s to ensure a stabilized fluorescence emission during the following F_M measurement. Then F_O was measured with a pulsed, blue measuring light (~900 μmol m^{-2} s^{-1}, 455 nm), and F_M was determined with a saturating white light pulse (~3000 μmol m^{-2} s^{-1}). The maximal quantum yield, F_V/F_M was calculated as $(F_M-F_O)/F_M$. To determine the chl a content of the alga, the alga (~0.5-0.6 g) was incubated in 5 mL of dimethylformamide (DMF) for 5 days at 4˚C in darkness. After 5 days, the absorbance of DMF extract was measured at 664.5 and 647

nm using DMF as blank. The chl a content was measured according to a formula by Inskeep and Bloom [10] (1). The value of the total chl content was in the unit of mg/g algal FW.

$$\text{Chl a (mg/L)}: 12.7*A_{664.5nm} - 2.79*A_{647nm} \quad (1)$$

Amino acid compositions were determined by using High-Performance Liquid Chromatography (HPLC), 1100 series (Agilent, USA). Algal samples were first ground in liquid nitrogen. About 10 mg of pulverized samples were then hydrolyzed in 2 mL of 6 M hydrochloric acid (HCl) in glass vials at 100°C for 24 hours. After 24 hours, 1 mL of borate buffer was added into the vial to dissolve the amino acids and filtered by using a 0.45 µm filter. Samples were then mixed with Bradford reagent to quantify its total soluble protein concentration at 595 nm. Remaining samples then were derivatized with *o*-phthalaldehyde reagent (5061-3355, Agilent, USA) at 50°C for 50 minutes. The derivatized sample was injected into the HPLC. The HPLC system was operated with amino acid column, Hypersil AA-ODS 2.1 x 200 mm 5u (Agilent, USA) at 0.7 mL/min flow rate, with 254 nm wavelength. The mobile phase consisted of two solvents (solvent A: 0.05 M ammonium acetate pH 6.8 and solvent B: 0.1 M ammonium acetate in 50% acetonitrile pH 6.8) with injection ratio of 70:30. The amino acids standard used was Amino Acid Standard from Agilent, USA (5061-3330).

Values of all the parameters tested were related to 100% of controls for better comparison except for accumulation of Hg and amino acids composition. Mean values and standard error were determined from three replicates of each treatment. The statistical significance of differences among means of different $Hg(NO_3)_2$ concentrations within similar species was calculated according to a Student's t-test. A probability level of $p<0.05$ was applied.

3. Results and discussion

It was observed that the amount of Hg in the tissues of all the algae is high (i.e. > 1000 mg/kg algae). Increasing the concentration of Hg from 3.1 to 6.2 µM, however, significantly reduced the ability of *Gracilaria salicornia* to accumulate Hg by 36% while *Ulva reticulata* showed >200% increment (Fig. 1a). *Sargassum* sp., on the other hand, did not show any changes in its ability to accumulate Hg. According to Baker and Brooks [11], plants that can be categorized as good metal accumulators are plants that can accumulate > 1000 mg/kg of metals. Thus, from the results obtained, it can be said that all the algae are good accumulators of Hg. The reduction in the ability to take in Hg may be due to the mechanisms used by *G. salicornia* are inhibited or deactivated. This theory is yet to be tested. Bioaccumulation is an active process occurs in living biomass which involves the mechanisms to concentrate the sorbents inside the biomass followed by transport of sorbents into inside of cells [7]. This process requires: (1) presence of nutrients, in this case the NO_3^{2-} bound to Hg (i.e. as $Hg(NO_3)_2$); and (2) cellular metabolic activity. In bioaccumulation, pollutants such as Hg are transported across the cell wall and membrane where inside the cells, will bound to intracellular structures. It was found that increasing the concentration of pollutant which is to be accumulated poses changes in morphology and physiology of cells [12]. If toxic metal ions are present within the cell, they pose toxicity which results from interactions of toxic metal ions with sulfhydryl groups of enzymes [7]. Thus, effects such as discussed below can be observed.

F_V/F_M is often used to indicate the influence of metals on the photosynthetic activity of the algae. The photosynthetic process of the algae was affected by the presence of Hg as shown by a decrease in F_V/F_M (Fig. 1b). In the presence of 3.1 µM $Hg(NO_3)_2$, F_V/F_M of the algae was reduced between 15 and 77%. However, in 6.2 µM, F_V/F_M of *Sargassum* sp. was significantly reduced to 11% while F_V/F_M of *G. salicornia* and *U. reticulata* was too low that the value was not detected by the fluorometer. At the physiological level, the measurement of F_V/F_M is an effective parameter to assess the photosynthetic status particularly the PSII of the alga under stress in which a reduction in this parameter indicates that the alga has been exposed to stress [3]. Measurement of F_v/F_m provide a first insight into changes of the photosynthetic apparatus upon the action of the metals [13] and can reveal the mechanisms involved in metals toxicity [14]. It is known that heavy metals could seriously affect the photosynthetic apparatus by irreversibly binding the components of photosynthetic electron transport chain. For instance, an increase in Hg content induces a significant increase in the proportion of the Q_B-non-reducing PSII reaction centers which is formed when the electron transfer from Q_A^- to Q_B is inhibited[2]. Lu et al. [2] also suggested that PSII reaction centers were the sites for Hg-induced damage. This suggestion was further supported by a study of

Kukarskikh et al. [15] which observed an increase in the steady-state level of P700 photo-oxidation indicating a disturbance in electron transfers between photosystems as well as an increase in fraction of closed reaction centers leading to reduction in non-photochemical quenching process. Hg is also able to alter the PSII machinery including the oxygen-evolving protein [1].

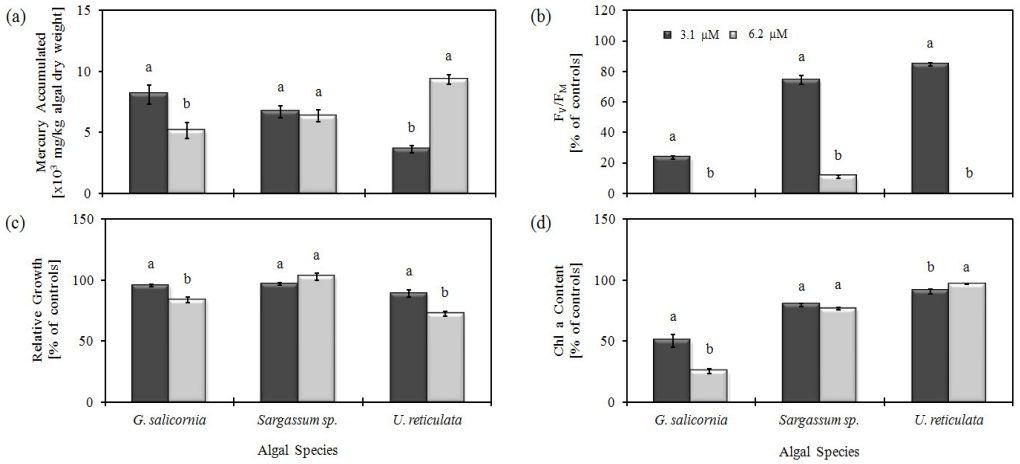

Fig. 1. (a) Amount of mercury accumulated in algal tissues, (b) maximal quantum yield, F_V/F_M, (c) relative growth, and, (d) chlorophyll (chl) a content of the macroalgae treated with 3.1 µM (*black bars*) and 6.2 µM (*grey bars*) $Hg(NO_3)_2$ for 8 hrs. Different letters above bars indicate statistically significant differences at $p<0.05$ between different Hg concentrations within similar algal species.

A significant loss of growth was observed in all the algae compared to untreated (Fig. 1c). A significant reduction was also observed in *G. Salicornia* and *U. reticulata* as the concentration of $Hg(NO_3)_2$ increases while the growth of *Sargassum* was not affected by this change (Fig.1c). Growth reduction could be due to blocking of cell division or elongation [16] or extra energy from metabolism may be needed by the cells to cope with the high accumulation of Hg [17]. In addition, Hg triggers oxidative stress that was responsible for the disturbances that lead to reduction in cell growth [18]. A red alga, *G. manilaensis*, when treated with Hg, was observed to trigger a different mechanism in detoxification of H_2O_2 by inducing a higher amount of catalase (CAT) than ascorbate peroxidase (APX) [19]. CAT is the main enzyme found in peroxisomes used to scavenge H_2O_2 generated during mitochondrial electron transport as well as β-oxidation of fatty acids [20]. Thus, it can be said that Hg may affect the respiratory as well lipid metabolic processes of alga.

The chl a content of all the algae was observed to be reduced as well (Fig. 1d). While there was a significant content decrease in *G. salicornia* as the concentration of $Hg(NO_3)_2$ increases, there was a significant increase in in *U. reticulata*. *Sargassum* sp., on the other hand, showed no changes between the two concentrations. Shakya et al. [21] and Rekha et al. [22] stated that heavy metals can inhibit chl biosynthesis resulting in the reduction of chl production. This effect is accomplished by the interaction of the metal with functional sulfhydryl groups of the enzymes in the chl biosynthetic pathway [22]. Hg has the ability to substitute magnesium ion (Mg^{2+}) at the centre of chl molecule, an important damage mechanism because it prevents the process of light harvesting which directly affects photosynthesis [23]. This may also explains the reduction in F_V/F_M observed for the algae (Fig. 1b). Contrastingly, a stimulation of chl a was observed by Janssen and Heijrick [24] at low concentration of metals. Bossuyt and Janssen [25] observed a significant increase in chl a at higher concentrations of metals. Zhang et al. [26] stated that significant increases in chl have been found to occur in response to a range of environmental stresses and are associated with stress resistance.

Table 1 shows the amino acids content of the algae after treatment with $Hg(NO_3)_2$ compared to untreated algae. A significant reduction in the total amino acids content was observed in *G. salicornia* and *Sargassum* sp. in both Hg concentrations compared to controls. *G. salicornia* showed a significant content increase while *Sargassum* sp. showed a significant content reduction as the concentration of Hg increases. In comparison, *U. reticulata* treated with 3.1 µM Hg showed a significant reduction in the total amino acids content compared to control but the value significantly increased from the control in 6.2 µM. Amino acids are constituents of proteins and proteins are

functional units of cell which are responsible for setting in motion the wide array of cellular events that occur in response to environmental changes. Metallothionein, a low molecular weight protein containing 30% cysteine (Cys), is synthesized by algae in response to heavy metals toxicity which can bind to metals and renders them inactive. Thus, this may explain the high amount of Cys observed for *U. reticulata* treated with 3.1 µM Hg (Fig. 2c). Newly formed histidine (His), glycine (Gly), arginine (Arg), serine (Ser), valine (Val), methionine (Met) and phenylalanine (Phe) in treated algae which are not initially found in the control algae (Fig. 2) may indicate the presence of new proteins that are high in these amino acids to help the algae in tolerating the toxicity of Hg as well as to compensate the need for energy to help in defense against Hg. These amino acids can be classified as either glucogenic or ketogenic which can donate intermediates for the citric acid cycle. Loss of amino acids such as aspartate (Asp), tyrosine (Tyr), Cys and threonine (Thr) in treated algae may indicate that Hg inhibits the synthesis of these amino acids. Since no further studies have been done on the effect of Hg on amino acids or proteins, these explanations are yet to be proven.

Table 1 Total amino acids content of the algae treated with two different concentrations of $Hg(NO_3)_2$ for 8 hrs compared to control. Data are mean±SE.

Algal Species	Total amino acids concentration [mg/mg algal FW]		
	Control	3.1 µM $Hg(NO_3)_2$	6.2 µM $Hg(NO_3)_2$
G. salicornia	118.9 ± 0.9^a	63.5 ± 2.6^c	103.8 ± 2.3^b
Sargassum sp.	122.4 ± 0.8^a	84.0 ± 0.6^b	79.0 ± 0.2^c
U. reticulata	168.5 ± 0.4^b	159.8 ± 1.8^c	237.8 ± 0.4^a

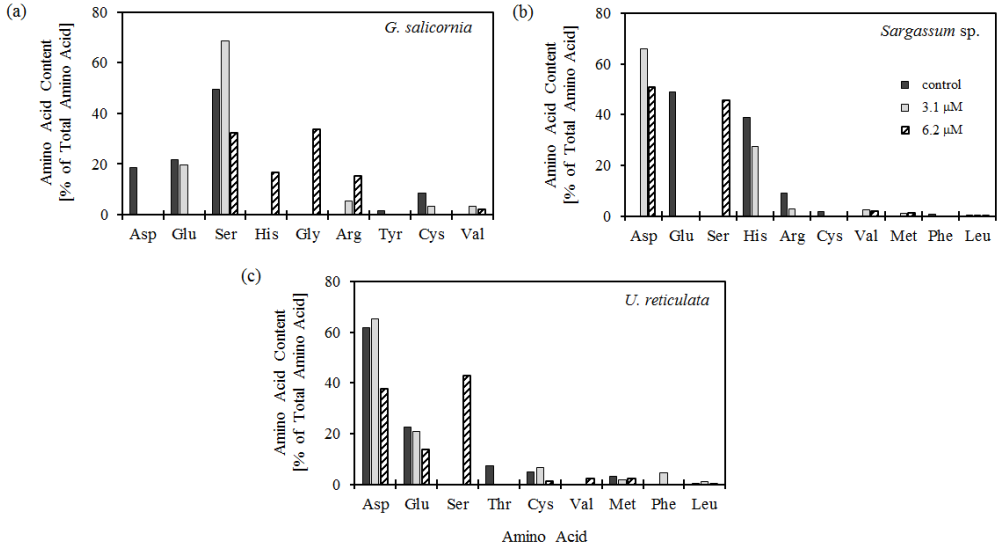

Fig. 2. Amino acid profile of the algae after treatment compared to untreated algae. (a) *G. salicornia*; (b) *Sargassum* sp.; (c) *U. reticulata*.

In conclusion, the algal studied showed different responses in the presence of Hg indicating that different mechanism was employed by the algae against the toxicity of Hg. High concentrations of Hg inhibits the photosynthetic efficiency of PSII and production of chl a leading to reductions in cell growth. Among the three algae studied, *G. salicornia* was the most affected while *Sargassum* sp. and *U. reticulata* can still tolerate Hg even at high concentration. PSII of *Sargassum* sp. is still functioning and *U. reticulata* synthesized more chl a at high Hg concentration. All algae have the ability to be used as used as bioremoval material but *U. reticulata* showed a much higher potential than the others. The different amino acids profile formed in treated compared to untreated algae showed that these amino acids can be used as biomarkers of Hg contamination. In addition, the changes in F_V/F_M and chl a content have the potential to be used as biomarkers as well.

Acknowledgements

The authors acknowledged the Malaysian Ministry of Education for funding the project under the Fundamental Research Grant Scheme (FRGS, Phase 2/2010) managed by the Research Management Center (RMC), UMT (vot. no. 59221).

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