PRELIMINARY STUDY ON THE POTENTIAL OF *GRACILARIA SP.* AS BIOREMEDIATOR OF METALS CONTAMINATION: THE DARK-ADAPTED QUANTUM YIELD AND CHLOROPHYLL A CONTENT

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Abstract: Algae-based biotechnologies for metals pollution control provide an attractive bioremoval method due to their ability to concentrate and tolerate high metal levels. This study is a preliminary study on the potential use of *Gracilaria* sp. as bioremediator of marine metals pollution. The effects of three metals (i.e. copper [Cu], lead [Pb], mercury [Hg]) on the dark-adapted quantum yield (i.e. F_v/F_m) and chlorophyll a (chl a) content of *G. edulis* and *G. manilaensis* were studied. The algae were exposed to 1 mg/mL of each of the metals individually for 8 h. The F_v/F_m of the algae was measured using a handheld fluorometer, AquaPen AP-P 100 (PIS, Czech Republic). Chl a of the algae were extracted in darkness at 4°C with dimethylformamide. F_v/F_m of both algae was decreased after the treatments except for *G. manilaensis* in Pb where it was unaffected. F_v/F_m of *G. edulis* was reduced significantly the most by Hg (48% of untreated algae), followed by Pb and Cu with more than 30%. F_v/F_m of *G. manilaensis* was reduced by both Cu and Hg with more than 23%. Interestingly, Cu and Pb did not have an effect on the chl a of *G. edulis* but a significant decrease of 34% was observed with Hg. A decrease in chl a was also observed in Cu for *G. manilaensis.* However, an increase in chl a was found in Pb and Hg for this alga. A reduction in F_v/F_m indicates that the algae were under metal stress except for *G. manilaensis* in Pb. The presence of Pb also seems to have a positive effect on *G. manilaensis* by increasing the chl a. An increase in chl a may also be an adaptive strategy of the alga to cope with the stress. Hg and Cu may have a phytotoxic effect on the chl a of *G. edulis* and *G. manilaensis*, respectively, which is confirmed by the F_v/F_m reduction. Further analysis would be done to confirm the results obtained in this study.

Keywords: Chlorophyll a, F_v/F_m, *Gracilaria edulis*, *Gracilaria manilaensis*, Heavy Metals.

INTRODUCTION

The increase in industrial activities has caused many water bodies receiving loads of heavy metals that exceed the maximum permissible limit for wastewater discharge designed to protect the environment [1]. Pollution by metal ions, including copper (Cu^{2+}), lead (Pb^{2+}) and mercury (Hg^{2+}), has become a major issue throughout many countries due to their possible toxic effects [2]. These metals are known to inhibit the photosynthetic process of plants and other autotrophs. Increased concentration of Cu, for instance, results in chlorosis and reduced growth of two brown algae [3]. Pb exposure damaged the structure and function of photosystem II (PSII) in the aquatic plant *Spirodela polyrrhiza* [4] while a decrease in photosynthetic pigments content, photosynthetic efficiency, chlorophyll integrity and cell membrane damage was observed in lichens exposed to Hg [5]. According to the Malaysian National Guidelines for Raw Drinking Water Quality established by the Ministry of Health, the benchmark for Cu, Pb and Hg was set up at 1.0 mg/L, 0.01 mg/L and 0.001 mg/L, respectively [6]. Sources of these toxic metals include metal smelters, effluents from plastics, textiles, microelectronics and wood preservatives producing industries and usage of fertilizer and pesticides [7]. These metals 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 [8].

Macroalgae (or seaweeds) play a major role in marine ecosystems. As the first organism in marine food chains, they provide nutrients and energy for animals. Moreover, beds of macroalgae provide shelter and habitat for scores of coastal animals for all or part of their lives. Macroalgae like any other plants 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 [9]. 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 [10]. These characteristics make them suitable for bioremediation process, a process which uses organisms to return the natural environment altered by pollutants or contaminants to its original state [11].

The aim of this study is to determine the effects of three heavy metals, Cu, Pb and Hg on two species of red alga *Gracilaria, G. edulis* and *G. manilaensis* in terms of their dark-adapted quantum yield, F_v/F_m and chl a content. This study is a preliminary study on the potential use of *Gracilaria* as a bioremediator as well as bioindicator of the three metals-polluted waters.

MATERIALS AND METHODS

Algal Materials

The algae, *Gracilaria edulis* and *Gracilaria manilaensis* were collected from the coast of Kuala Muda, Kedah, Malaysia and further cultivated at the Seawater Hatchery, Universiti Malaysia Terengganu in open tank system. Prior to analysis, the algae were cleaned to get rid of unwanted materials or parasites.

Heavy Metals Treatments

About 5 g of the algae were treated with 0.001 mg/L each of copper(II) nitrate ($Cu(NO_3)_2$), lead(II) nitrate ($Pb(NO_3)_2$) and mercury(II) nitrate ($Hg(NO_3)_2$) for 8 h in aerated beakers under white light. The concentration of metals used was higher than that of the Class 2 Malaysian Island Marine Water Quality Status set by the Department of Environment which is 2.9 µg/L for Cu, 8.5 µg/L for Pb and 0.16 µg/L for Hg [12]. The conditions for the controls or untreated algae were similar to the treated algae but without addition of metals. Each experiments were done in triplicates.

Chlorophyll a Fluorescence Measurement

Chlorophyll a fluorescence was measured with a handheld chlorophyll fluorometer, AquaPen-P AP-P 100 (Photon Systems Instruments, Czech Republic). The dark-adapted quantum yield (i.e. F_v/F_m) of the algae was measured according to that of the manufacturer's operating manual. The ratio F_v/F_m was determined before and immediately 8 h after the treatment (F_m = maximal fluorescence; F_0 = initial fluorescence; variable fluorescence, $F_v = F_m - F_0$).

Chlorophyll a Content Determination

Chlorophyll (chl) a was extracted in 5 mL of dimethylformamide (DMF) for 5 days at 4˚C in darkness [13]. After 5 days, the absorbance of the DMF extracts was measured at 664.5 and 647 nm using DMF as blank. The chl a content was measured using the formula as shown in Eq. (1) [14].

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

Statistical Analysis

Values of all the parameters tested were related to 100% of control (i.e. untreated algae) for better comparison. Mean values and standard deviation were determined from three replicates of each treatment. The statistical significance of differences among means was calculated according to a one-way ANOVA followed by Fischer's least significance difference (LSD) test. A probability level of $P<0.05$ was applied.

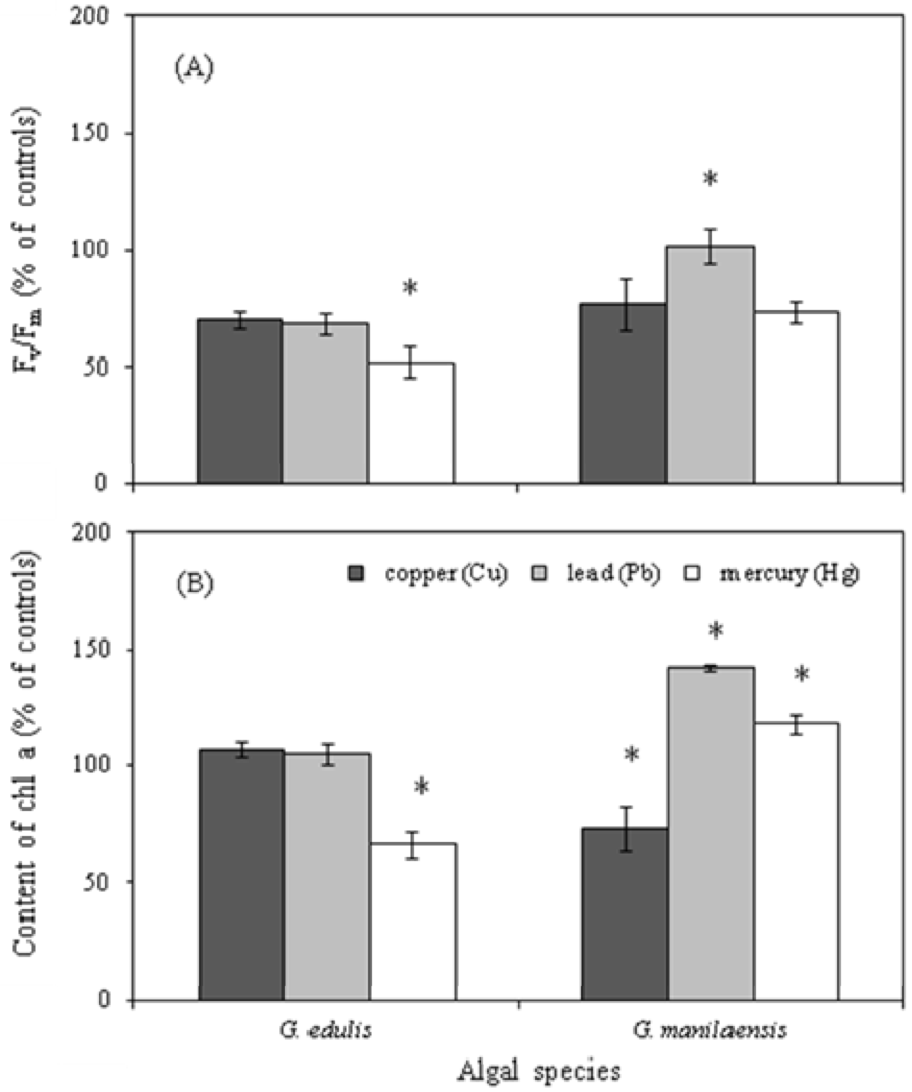

Figure 1: The dark-adapted quantum yield (F_v/F_m, *A*) and chlorophyll a content (*B*) of *G. edulis* and *G. manilaensis* after 8h treatment with the heavy metals. Asterisk above bars indicate statistically significant difference between metals within similar species (ANOVA, Fischer's LSD test, $P<0.05$, n=3).

RESULTS AND DISCUSSION

The dark-adapted quantum yield (i.e. F_v/F_m) of both the algae was affected by the heavy metals presence with one exception (Fig. 1a). F_v/F_m of *Gracilaria edulis* in copper (Cu) and lead (Pb) was reduced to more than 30% while F_v/F_m of this alga was significantly lower in mercury (Hg) than the other two metals with a reduction of more than 48%. F_v/F_m of *Gracilaria manilaensis* on the other hand, was not significantly affected by Pb but was reduced to more than 23% in Cu and Hg. According to Maxwell and Johnson [15], F_v/F_m can be used as a sensitive indicator of plant's photosynthetic performance. A reduction in F_v/F_m is often used to indicate stress in plants which frequently occurred when plants are exposed to abiotic and biotic stresses in the light [16]. Thus, the reduction in F_v/F_m of both the algae (with one exception) in this study may indicate that the algae were stressed by the presence of metals. The metals may in some ways disturb or damage the photosynthetic apparatus of the algae resulting in the decline in the quantum yield of photosystem II (PSII) photochemistry [17]. Toxic effects of metals appear to be partly related to the production of reactive oxygen species (ROS) as well, which can cause oxidative damage to cells [18]. A decrease in F_v/F_m in the presence of heavy metals has been observed in other marine macroalgae including *Ulva pertusa* and *Ecklonia cava* [19], and, *Palmaria palmata, Chondrus crispus*, *Ascophyllum nodosum* and *Cladophora rupestris* [20]. Interestingly, F_v/F_m of *G. manilaensis* was not affected by Pb. Pb^{2+} is known to accumulate in PSII and damage its secondary structure while decreasing the absorbance of visible light, inhibited energy transfer within the PSII protein–pigment complex, and reduced energy transport to chl a [4]. However, in a study by Baumann et al. [20] on seven species of marine macroalgae, it was observed that Pb did not have an effect on the fluorescence yield indicating that the algae were tolerant to Pb. In addition, these algae had accumulated higher concentration of Pb which suggests that the algae would be good organisms for use in bioremediation of Pb-polluted waters. Thus, *G. manilaensis* in this study is also tolerant to Pb and can be a good candidate for use as bioremediator of Pb.

In comparison to F_v/F_m, chlorophyll a (chl a) content of the algae did not reflect the fluorescence yield. A significant reduction in the content of chl a was observed for *G. edulis* in Hg (34% reduction) and *G. manilaensis* in Cu (27% reduction) (Fig. 1b). Heavy metals have been known to inhibit chl biosynthesis resulting in the reduction of chl production [21-22] which can be accomplished by the interaction of the metal with functional sulfhydryl (-SH-) groups of the enzymes in the chl biosynthetic pathway [22]. High concentrations of Cu may also induce oxidative damage that can alter the cell membrane properties thereby demonstrating the inhibitory effect on the enzymes involved in chl production [21]. Cu and Hg which have the ability to substitute magnesium ion (Mg^{2+}) at the centre of chl molecule, is an important damage mechanism because it prevents the process of light harvesting directly affecting photosynthesis [23-24]. This may also explains the reduction in F_v/F_m observed for both the algae (Fig. 1a). Contrastingly, chl a of *G. manilaensis* in Pb and Hg was significantly increased 42% and 18%, respectively. The stimulation of chl a was also observed by Knauer et al. [25] and Janssen and Heijrick [26] at low concentration of metals while Bossuyt and Janssen [27] who found a significant increase in chl a at higher concentrations of metals in a freshwater green alga *Pseudokirchneriella subcapitata*. Soto et al. [28] on the other hand, found that the chl a is increased at lower concentration of Cu but decrease when the concentration is increased. The increase of chl a was observed in the green alga *Ulva armoricana* exposed to 100 µg/L Cu with no reduction in F_v/F_m but a decrease in relative growth rate [29], This supports the idea that there was an exchange between energetic resources being used for pigment biosynthesis and growth [29]. This could also be the case for *G. manilaensis* exposed in Pb observed in this study which increased in chl a (Fig. 1b) but with no change in F_v/F_m (Fig. 1a) and a decrease in weight (data not shown). More generally, significant increases in chl have been found to occur in response to a range of environmental stresses and are associated with stress resistance [30]. The disparity between F_v/F_m and chl a content observed may also be that chl a affected was mostly the component of photosystem I (PSI) since PSI was consists entirely of chl a while chl fluorescence only probes the PSII [29].

CONCLUSIONS

In conclusion, the heavy metals investigated in this study demonstrate a range of significantly different stress effects. While reducing the photosystem II efficiency of the algae, the metals induce a different pattern on the chlorophyll a (chl a) content. An increase in chl a content may indicate some kind of adaptive strategy, while a reduction may indicate damage to the molecular structure or interference in its biosynthetic pathway. A reduction in F_v/F_m on the other hand, may indicate stress in the algae with damage or interference to the photosystem II or any other components of photosynthetic apparatus. Both of the algae also showed different responses. *G. manilaensis* was able to adapt to Pb but not with other metals while *G. edulis* was negatively affected by the three metals. However, further analysis will be done in order to understand more about the underlying mechanisms that generate these results and finally to be able to find which of these two algae is the best bioremediator for copper-, lead- and mercury-polluted waters. In future, proteomics analysis, morphological study and bioaccumulation analysis will be done on the algae in responses to various concentrations of these three metals.

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