Effects of Varying Copper Concentrations on Photosynthesis of *Gracilaria salicornia* and *Padina sanctae-crusis*

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Two tropical macroalgae, *Gracilaria salicornia* and *Padina sanctae-crusis*, were exposed to varying concentrations of total Cu and subsequently subjected to photosynthesis-irradiance (P-I) response experiments. The study aimed to determine the effects of total Cu toxicity on the P-I model parameters and growth rate of the macroalgae. The photosynthetic efficiency (α) showed a decreasing pattern with increasing total Cu concentration. Light saturation (I_k) for both algae increased at lower concentration and showed transient shift at 12.5 µg total Cu L^{-1}. The maximum photosynthesis (P_{max}) of *P. sanctae-crusis* was higher in specimens exposed to 12.5-25 µg total Cu L^{-1} compared to the controls. Unlike *P. sanctae-crusis*, *G. salicornia* exposed total Cu-free medium as well as those at 12.5-25 µg total Cu L^{-1} were comparable. But, both algae showed decreasing P_{max} values from 50-500 µg total Cu L^{-1}. Respiration (R) showed non-linear pattern due to some delay of the effect of copper on the respiratory system. Growth data proved to be more sensitive to total Cu with the reductions of mean daily growth rate starting at 12.5 µg total Cu L^{-1} for *G. salicornia* and negative growth rate at 500 µg total Cu L^{-1} for *P. sanctae-crusis*. After 7 days of exposure the EC_{20} for *G. salicornia* and *P. sanctae-crusis* were 100 and 50 µg total Cu L^{-1}, respectively. The results suggested severe impact of total Cu^{+} at high concentrations on P-I parameters and growth rate of *G. salicornia* and *P. sanctae-crusis*.

Key words: coral reefs, macroalgae, Phaeophyta, photoinhibition, photosynthesis-irradiance, Rhodophyta

INTRODUCTION

Copper (Cu) is an essential micronutrient for all photosynthetic organisms (i.e. cyanobacteria, algae, and plants) and plays an important role in numerous metabolic and physiological processes (Bernal et al. 2006). However, at elevated concentrations, Cu can inhibit a large number of enzymes and can interfere with several aspects of plant biochemistry, including photosynthesis, pigment synthesis, and membrane integrity (Fernandes

and Henriques 1991). In off-shore seawater, ambient total dissolved copper (II) concentrations range from 0.069-0.693 µg L^{-1} but levels of total Cu may be far higher (64-2,029 µg L^{-1}) in coastal and estuarine waters, due to both natural and anthropogenic origins (Brown and Newman 2003; Hill 1999). In 1996, Marcopper mine on the island of Marinduque, Philippines spilled three million metric tons of tailings and were released into the Boac River. In 2012, Philex Padcal mine in Benguet, Philippines spilled 20 million metric tons of mineral tailings and were drained into the Balog and Agno River

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systems. These incidences involving waste spillover lead to siltation and contamination of rivers and coastal areas (Holden 2015; Raymundo 2014).

Detailed investigations on the effects of copper on photosynthesis of planktonic microalga *Phaeodactylum tricornutum* have been done previously (Cid et al. 1995). The main reasons for selecting planktonic algae as test organisms are the relative ease with which they can be handled in the laboratory and, perhaps more importantly, their short generation time (Hunding and Lange 1978). However, coastal and estuarine waters are more polluted than the oceans since the waste product that has been introduced in the rivers of the industrial and population centers will most likely be released into these areas (Li et al. 2000). These areas are dominated by benthic macroalgae which are the major contributor of net primary production (Carmen et al. 2009). Macroalgae also release significant quantities of dissolved and particulate organic matter to coastal waters and are grazed directly by herbivores (Ramus 1992). Among macroalgae, the Rhodophyta (red algae) and Phaeophyta (brown algae) are the ones containing a large assemblage of species that predominate the coastal areas of tropical, temperate, and cold-water regions (Kumar et al. 2008; Ortiz and Trono 2000). Rhodophyta is a distinct eukaryotic lineage with over 90 % of them having chlorophyll *a* and accessory photosynthetic pigments like carotenoids, phycoerythrin, and phycocyanin (Woelkerling 1990). Conversely, the major photosynthetic pigments of Phaeophyta are chlorophyll *a* and *c* plus varying amounts of fucoxanthin, *β*-carotene, violaxanthin, and diatoxanthin (Wehr 2002).

In the Philippines, the red alga *Gracilaria salicornia* Agardh and the brown alga *Padina sanctae-crusis* Børgesen are common in shallow-waters (Geraldino et al. 2005). Despite their abundance and widespread distribution, little is known about their ecophysiology, especially their conditions upon total Cu exposure. With a growing interest on *Gracilaria* sp. and *Padina* sp. as potential candidates for nutrient removal of wastewater treatment and as bioremediation agents (Dhargalkar and Pereira 2005), knowledge about the effects of total Cu on these algae and methods to assess its adverse effect are thus needed to provide baseline information. A sound and empirically-derived physiological knowledge of *G. salicornia* and *P. sanctae-crusis* will provide the framework against which their economic and ecological utilization can be determined.

Photosynthesis-irradiance (P-I) curve plays a central role in modeling, measuring, and predicting physiological changes such as photosynthesis, and in assessing both intra and interspecific variations in photosynthetic physiology (Mishra 2004). The parameters obtained in this curve contain important information on the functioning of various components of the photosynthetic apparatus and their response to environmental variables. Studies on the use of P-I curves for toxicity testing of marine macrophytes are widely scattered and focused on *Fucus* sp. and *Ulva* sp. (Xu et al. 2014; Binzer and Sand-Jensen 2002; Henley 1993). Comparatively, few P-I studies were done on *G. salicornia* and *P. sanctae-crusis* (Cox and Smith 2015; Phooprong et al. 2007). In the Philippines, despite harboring highly diverse assemblage of macroalgae, no P-I study has been done in conjunction with toxicity testing, although some employed this method in their studies on macroalgal ecophysiology (Ganzon-Fortes et al. 2009). In this context, the effects of varying total copper concentrations (control, 12.5, 25, 50, 250, 500 μg total Cu L^{-1}) on the P-I parameters (i.e., initial slope (α), saturating irradiance level (I_k), maximum photosynthesis (*Pmax*), respiration (*R*)) and the mean daily growth rate of *G. salicornia* and *P. sanctae-crusis* were determined.

MATERIALS AND METHODS

Sampling and acclimation of algae
Healthy and epiphyte-free thalli of *Gracilaria salicornia* and fronds of *Padina sanctae-crusis* were collected in Cordova, Mactan Island, Central Philippines (Figure 1). *G. salicornia* can be distinguished from the others through its di- to trichotomous branching and irregularly constricted axes (Iyer et al. 2004). On the other hand, *P. sanctae-crusis* is distinguishable through its light-brown colored thallus that is heavily calcified on both surface (Geraldino et al. 2005). Collected algae were packed in mesh bags, immersed in natural seawater, and were brought to the laboratory at the University of San Carlos - Marine Station, Maribago, Mactan Island. The algae were acclimatized in an environmental chamber for 24 hours. The algae were placed in small jars containing GFC filtered seawater, maintained at constant temperature of 24°C and a photosynthetic photon fluence rate (PPFR) of 50 μmol m^{-2} s^{-1} with adequate aeration in a 12:12 light:dark cycle.

Total Cu stock solution and incubation experiment
Copper sulphate (in the form of $CuSO_4.5H_2O$), a compound commonly applied in copper toxicity testing was used in the preparation of stock solution (Nielsen and Nielsen 2010; Perales-Vela et al. 2007). Copper sulphate was chosen because it is listed by United States Environmental Protection Agency as a reference substance that produces consistent results in evaluating the quality of test organisms and analyzing its sensitivity (Viana and Rocha 2005). Analytical grade and oven dried $CuSO_4.5H_2O$ (3.9 mg) were dissolved in distilled water
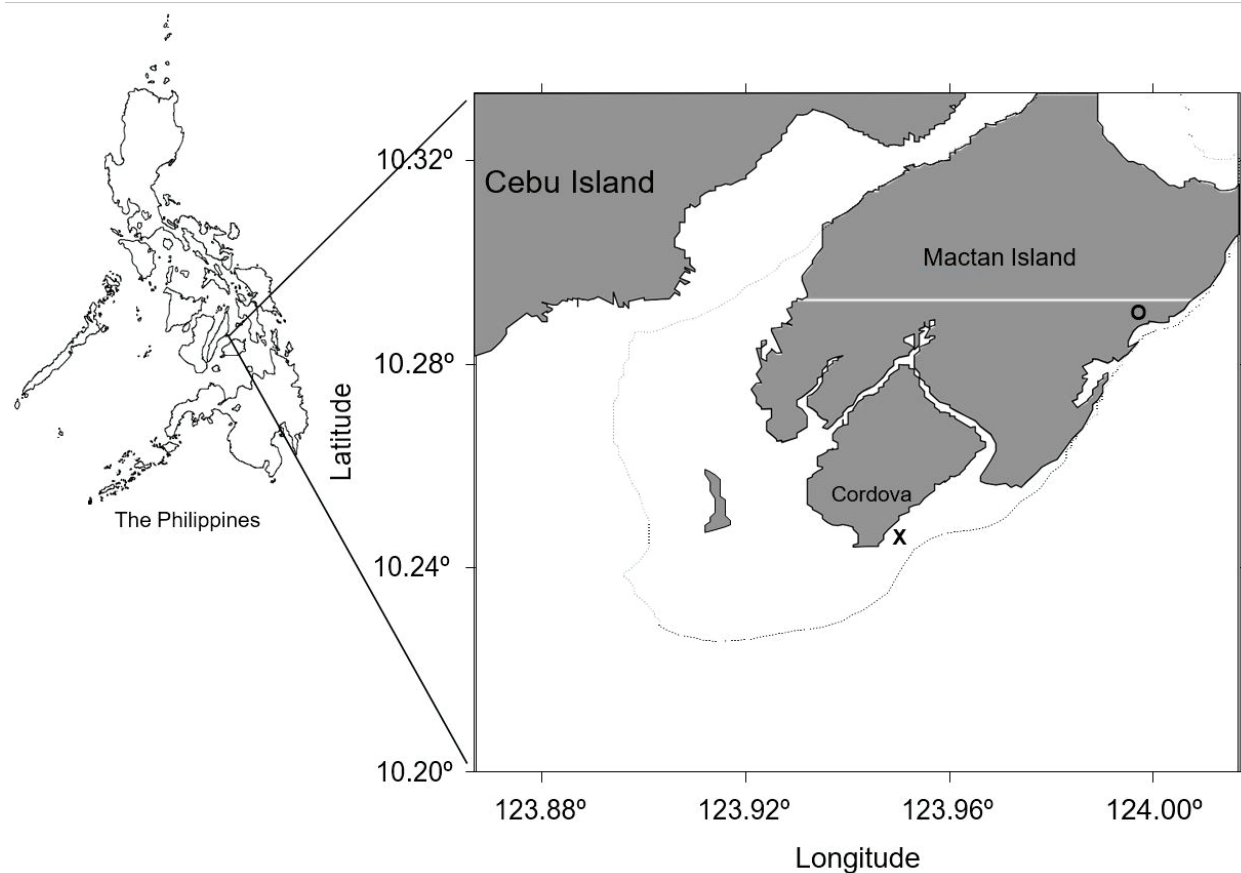
Figure 1. Sites where the macroalgae were collected (Cordova marked as **X**) and where the experiment was conducted (University of San Carlos marine station marked as **O**) in Mactan Island, Central Philippines.

and made up to 1 L to come up with 1 mg L^{-1} total Cu stock solution. Subsequently, working total Cu concentrations were made from the stock solutions based on ratio and proportion. GFC filtered seawater was used as diluents for preparing test solutions including the controls. Hence, the working concentrations can be considered above ambient concentrations.

Six thalli of *G. salicornia* and fronds of *P. sanctae-crusis* each 2-g wet weight were excised from different individuals and placed in 2-L glass container provided with an aerator. Each jar was covered with a cellulose membrane to avoid excessive evaporation. Temperature (°C), pH, and salinity were monitored daily. The seawater media were changed everyday for seven days to replenish the naturally occurring nutrients and total Cu concentrations (12.5, 25, 50, 250, 500 µg total Cu L^{-1}) and remove exudates.

After seven days of Cu exposure, the photosynthetic responses of algae to different light intensities were assessed. Thallus from a specific treatment and replicate was randomly assigned to one of the six incident light intensities (i.e. 0, 200, 400, 600, 800, 1000 µmol m^{-2} s^{-1}).

The six varying light intensities were achieved using a modified junction box with five 80 watts PAR 38 spot bulbs. The algal specimens were placed in biological oxygen demand (BOD) bottles (volume=237 mL) containing filtered seawater, and were incubated in a rotating plexiglass incubating chamber for one hour. The initial and final dissolved oxygen concentrations after incubating algae were determined using a dissolved oxygen (DO) meter (YSI model 58; precision = 0.01 mg L^{-1}). Rates of photosynthesis were calculated as the difference between the initial and final DO concentration multiplied by the volume of the BOD bottles divided by the dry weight of the algae and the incubation time. Computations were standardized to mg O_2 g^{-1} dry weight-h^{-1}. Overall, there were 432 photosynthesis measurements representing two species of algae, six levels of total Cu treatment (including controls), six replicates per species per total Cu treatment, and six levels of irradiances (including dark respiration).

Determination of Daily Growth Rate and EC$_{50}$ and EC$_{20}$ values

During the P-I response pretrial experiment, the thin outer layer of the fronds of *P. sanctae-crusis* treated with higher concentration of total Cu were observed to peel. This could alter the dry weight (DW) and could become a possible source of error in computing the net photosynthesis. To prevent this from happening, the DW and wet weight (WW) relationship was established. Untreated *P. sanctae-crusis* were weighed according to six common predetermined WW of the fronds. A total of 18 points (i.e. WW and DW pairs) were made for the six WW. The fronds were placed in an oven at 60°C for 24 hours and immediately transferred to a desiccator with silica gel for 48 hours. The DW was then estimated. The relationship between DW and WW of the *P. sanctae-crusis* was determined by linear regression.

The growth rate of algal biomass [Eq. 1] and the seven days growth inhibition [Eq. 2] were determined according to OECD test guideline 201 (OECD 2002) using the following formula:

$$DGR = (\ln W_2 - \ln W_1)/(t_2 - t_1) \qquad [Eq.1]$$

$$IGR = [(GR_C - GR_T)/GR_C] \times 100 \qquad [Eq. 2]$$

where DGR represent the mean daily growth rate, W_2= fresh WW at t_2, W_1= initial fresh weight, t= time in days, IGR= inhibition of growth rate, GR_C= mean growth rate of the algae at control, and GR_T= mean growth rate of the total Cu treated algae.

In this study, EC$_{50}$ and EC$_{20}$ were defined as concentrations of total Cu that caused decrease in growth to 50% and 20% relative to the control, respectively. These were estimated according to Mamboya et al. (2009) wherein a dose-response diagram was plotted using growth rate as percent of the controls' growth rates versus logarithm to base ten of the total Cu exposure concentrations. A regression line was fitted.

Model comparison and assessment of P-I model parameters

The functions of describing control of photosynthesis by light are generally defined to reproduce the usual shape of P-I curve, which implies that it must have zero primary production at zero irradiance. Two equations (or models) that can produce a sensible value to the raw P-I data were selected among the most widely used expressions for the light saturation function namely:

$$\text{Model 1: } P = ((\alpha * I) * (1 - ((\alpha * I)/\beta))) + R \qquad [Eq. 3]$$

$$\text{Model 2: } P = ((\alpha * I)/(\beta + I)) + R \qquad [Eq. 4]$$

where P is photosynthesis (mg O^2 g^{-1} DW-h^{-1}), I is irradiance µmol m^{-2} s^{-1}. α and β are initial parameters.

Eq. 3 is the Jassby and Platt (1976) model while Eq. 4 is the hyperbolic model in Cosby et al. (1984). The two equations described α and P_{max} which represent the maximum light utilization coefficient and the maximum photosynthetic rate, respectively. The two equations were presented in equivalent form and may legitimately be compared with respect to their success in fitting experimental data.

The fit of the P-I curve against the P-I measurements was carried out using the Levenberg-Marquardt nonlinear least square regression technique. P-I parameters such as maximum photosynthesis (P_{max}), initial slope (α), saturating irradiance level (I_k), and respiration (R) were derived after fitting the equation. The two equations were then compared based on their goodness of fit (R^2 and SSR) with the P-I data. Additionally, the second order Akaike Information Criterion (AIC$_c$) of Akaike (1981) was used to confirm and to make sure which of the two models has a better fit. The model with the lower AIC$_c$ score was likely to be correct and was subsequently used.

Statistical Analysis

A two-way nonparametric ANOVA was used for each model parameter *in lieu* of a Model I two-way factorial ANOVA with replicates since the P-I parameter data consistently violate the assumptions of parametric ANOVA. The data were ranked from smallest to largest, and a two-way ANOVA on the ranks was carried out. The Scheirer-Ray-Hare extension of the Kruskall-Wallis test was done. The significance level for all tests was set at P= 0.05. Dunn test was used (Zar 2010) for multiple comparison if there was significance in at least one of the main factors after the two-way nonparametric ANOVA test.

RESULTS

The DW and WW relationship of *P. sanctae-crusis* correspond to the equation DW= 0.192x + 0.011, where DW is the dry weight (g) and x is the WW (g) (Figure 2). The fit of the linear regression model for the two variables was highly significant (R^2= 0.96, p<0.001). Hence, DWs of *P. sanctae-crusis* were computed based on the said equation before computing for photosynthesis.

Growth response to copper

The mean daily growth rate in the absence of total Cu was 0.02 g WW d^{-1} for G. salicornia and 0.06 g WW d^{-1} for *P. sanctae-crusis* (Figure 3). There were significant differences in, and interaction between the main factors (total Cu concentration and algal type) (Table 1). For *P. sanctae-crusis*, Cu started to inhibit the algal growth at 12.5 µg total Cu L^{-1}, but at 25 µg total Cu L^{-1}, a non-significant stimulative effect compared to 0 and 25 µg L^{-1}
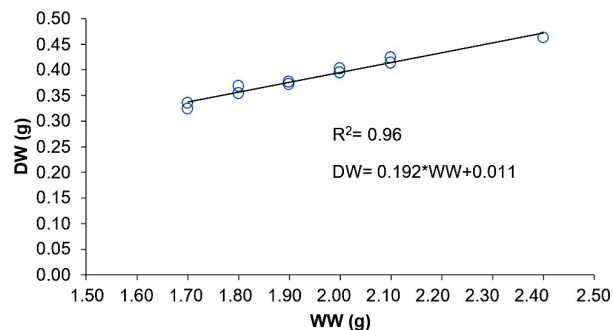
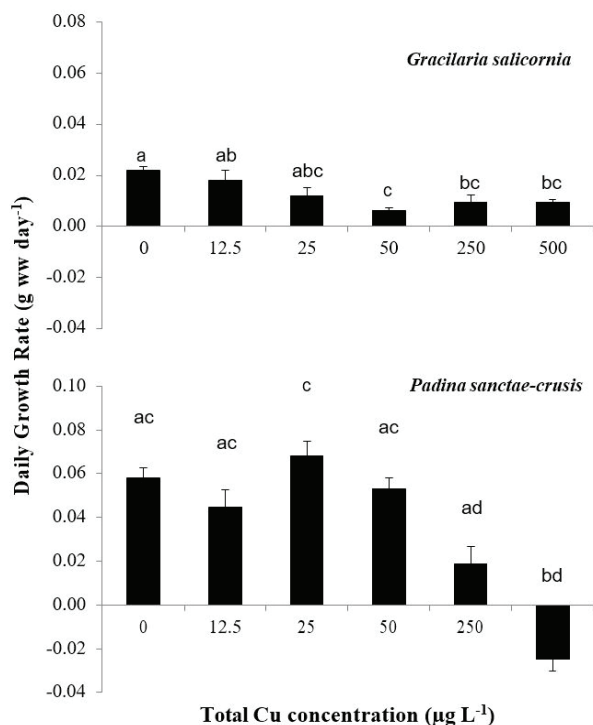**Figure 2.** DW-WW (g) relationship of *Padina sanctae-crusis*.

Figure 3. Daily growth rate (mean±s.e.,n=6) of the two macroalgae *Gracilaria salicornia* and *Padina sanctae crusis* exposed to different total Cu concentrations for 7 days. Two columns sharing the same letter are not significantly different.

Table 1. Statistical results on the effect of total Cu concentrations and macroalgae species on P-I parameters (α, Ik, $Pmax$, R) and daily growth rate (DGR).

P-I parameters	Effects	SS	df	H	p-value
α	Total Cu Conc.	16693.7	5	38.2	0.00*
	Algae	20.1	1	0.0	0.83
	Total Cu Conc *Algae	1715.1	5	3.9	0.56
	Error	12626.2	60		
I_k	Total Cu Conc.	9990.8	5	22.8	0.00*
	Algae	1568.0	1	3.6	0.06
	Total Cu Conc *Algae	186.2	5	0.4	1.00
	Error	19352.5	60		
P_{max}	Total Cu Conc.	19456.8	5	44.4	0.00*
	Algae	2787.6	1	6.2	0.01*
	Total Cu Conc *Algae	2328.7	5	5.3	0.38
	Error	6523.9	60		
R	Total Cu Conc.	5400.3	5	14.9	0.02*
	Algae	1225.1	1	0.2	0.09
	Total Cu Conc *Algae	2809.0	5	6.9	0.27
	Error	21662.2	60		
DGR	Total Cu Conc.	10552.0	5	24.1	0.00*
	Algae	4640.1	1	10.6	0.00*
	Total Cu Conc *Algae	5963.2	5	13.6	0.02*
	Error	9912.3	60		

*significant at $p<0.05$; α–photo synthesis efficiency; I_k-light saturation; P_{max}-maximum photosynthesis; R-respiration

can be observed. A pronounced decrease in the algal growth rate can be seen at 50, 250, and 500 µg total Cu L^{-1} with inhibitions of 8.25 %, 67.44 %, and 142 % respectively. For *G. salicornia*, a decreasing daily growth rate with increasing total Cu concentration was observed. At lower concentrations (12.5 and 25 µg total Cu L^{-1}), growth rate inhibition were already noticeable. The highest inhibition (27 %) occurred at 500 µg total Cu L^{-1}.

The seven days EC$_{20}$ for *G. salicornia* and *P. sanctae-crusis* were 100 and 50 µg total Cu L^{-1}, respectively (Figure 4). On the other hand, the EC$_{50}$ for *P. sanctae-crusis* was 80 µg total Cu L^{-1} while that for *G. salicornia*

could not be derived using the current data.

Aside from the significant effect of total Cu concentration on the growth rate and P-I parameters, other notable changes were observed at 50 to 500 µg total Cu L^{-1} for *P. sanctae-crusis*. The culture media turned from colorless to light yellow after 24 hours exposure and the thin outer layer of the fronds started to wear off after four days of exposure to 500 µg total Cu L^{-1}. For *G. salicornia*, the color of the thalli became translucent on the 7th day of exposure to 500 µg total Cu L^{-1}.
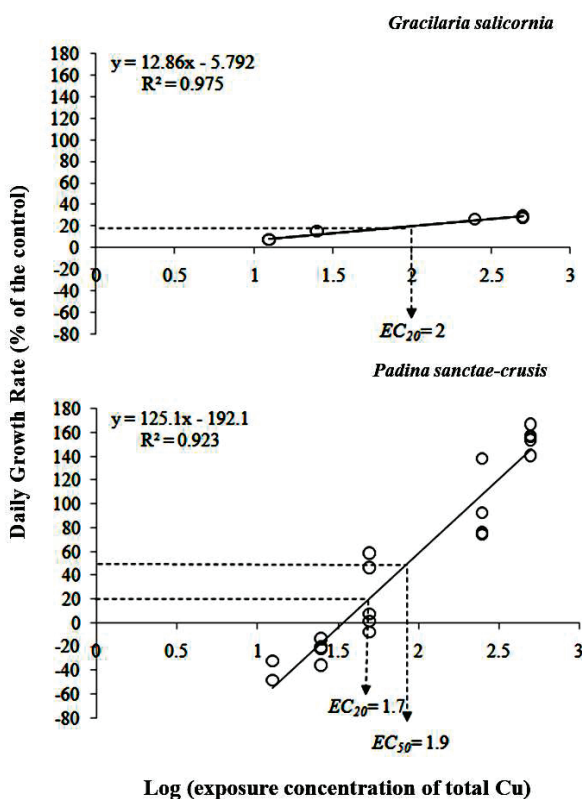
Figure 4. Growth dose-response diagram of the two macroalgae *Gracilaria salicornia* and *Padina sanctae crusis* exposed to different total Cu concentrations for 7 days. The open circles are individual replicates. EC is the concentration of total Cu that caused a decrease in growth equivalent to 20 or 50 % of the control.

Photosynthesis response to Copper

Model 2 [i.e. $P=((\alpha*I)/(\beta+I))+R)$] showed a higher R^2 and lower SSR values than Model 1. The results generated using AIC_c were also analyzed: the value of Model 2 was 14.04 and is 1.13 times more likely to be correct than Model 1 for all data sets. Model 2 was thus used to derive all P-I parameters.

The P-I curves of *G. salicornia* and *P. sanctae-crusis* showed a fast initial rise, with increasing light intensity up to about 200 µmol m^{-2} s^{-1}, then eventually became light saturated and remained unchanged with additional light intensity (Figures 5a and 5b). In some algal samples, photoinhibition occurred at 1000 µmol m^{-2} s^{-1}. There was no significant interaction between total Cu concentrations and algal species on all P-I parameters (Table 1). *G. salicornia* and *P. sanctae-crusis* showed comparable initial slope (α) (Figures 6 and 7). But, the effect of total Cu concentration was significant and showed lower values against the control, with both species displaying a decreasing trend with an increasing total Cu concentration. The trend was visibly pronounced in *G. salicornia*.
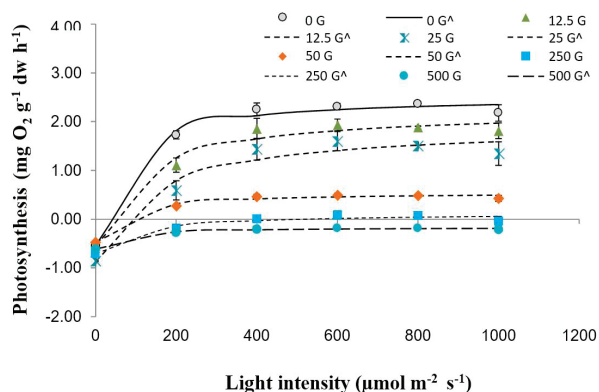

Figure 5a. Net photosynthesis (mean±s.e.,*n*=6) of *Gracilaria salicornia* across irradiance levels after subjecting to different concentration of total Cu (G=Observed, G^=Predicted). The data were fitted using the model, $P=((\alpha*I)/(\beta+I))+R)$.
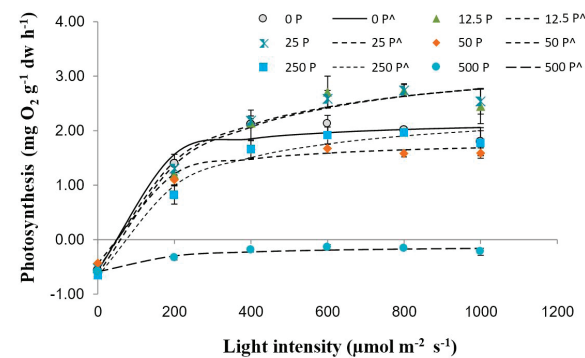

Figure 5b. Net photosynthesis (mean±s.e.,*n*=6) of *Padina sanctae-crusis* across irradiance levels after subjecting to different concentration of total Cu (P=Observed, P^=Predicted). The data were fitted using the model, $P=((\alpha*I)/(\beta+I))+R)$.

For both algae, the level of irradiance at which saturation occurred (I_k) differed among total Cu concentrations ($P<0.05$; Figures 6 and 7). The effect of increasing total Cu concentrations on I_k appeared to be bimodal with higher values at mid-total Cu concentration and another value at extreme total Cu concentration. However, the *post-hoc* Dunn test revealed statistically significant differences between 0 and 25 and between 25 and 500 µg total Cu L^{-1} for *G. salicornia*. The same was observed between 0 and 12.5, 0 and 25 µg total Cu L^{-1}, and between 25 to 50 µg total Cu L^{-1} for *P. sanctae-crusis*.

The pattern of maximum production (P_{max}) varied at different total Cu concentrations for both algae. *G. salicornia* had the highest P_{max} of 3.1 at 0 µg total Cu L^{-1} and *P. sanctae-crusis* had the highest P_{max} of 4.6 at 12.5 µg total Cu L^{-1} (Figures 6 and 7). For *G. salicornia*, P_{max} declined with increasing total Cu concentrations. However, for *P. sanctae-crusis*, the trend of P_{max} appeared
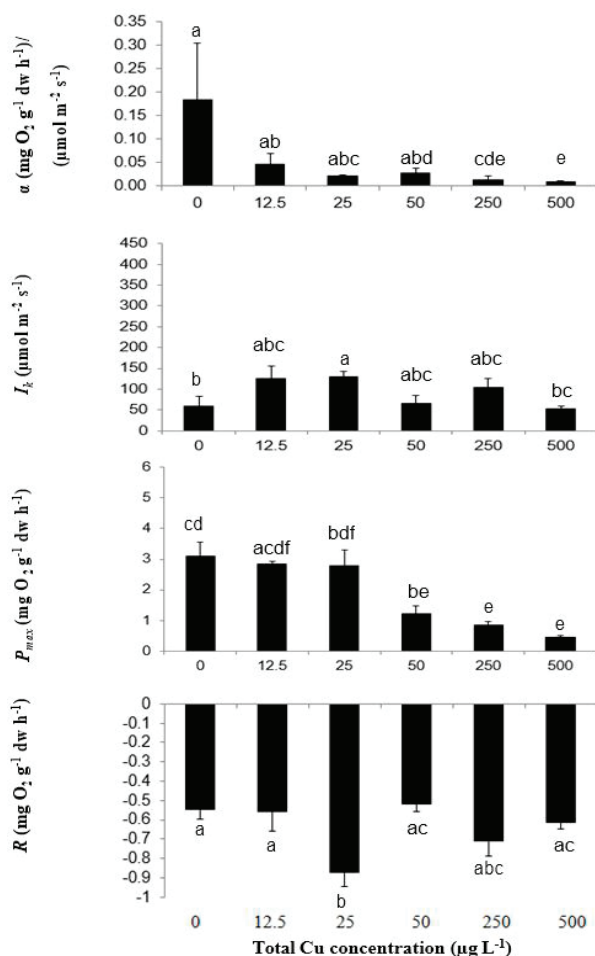
Figure 6. Photosynthesis-Irradiance parameters (mean±s.e.,*n*=6) of *Gracilaria salicornia*. Two columns sharing the same letter are not significantly different.
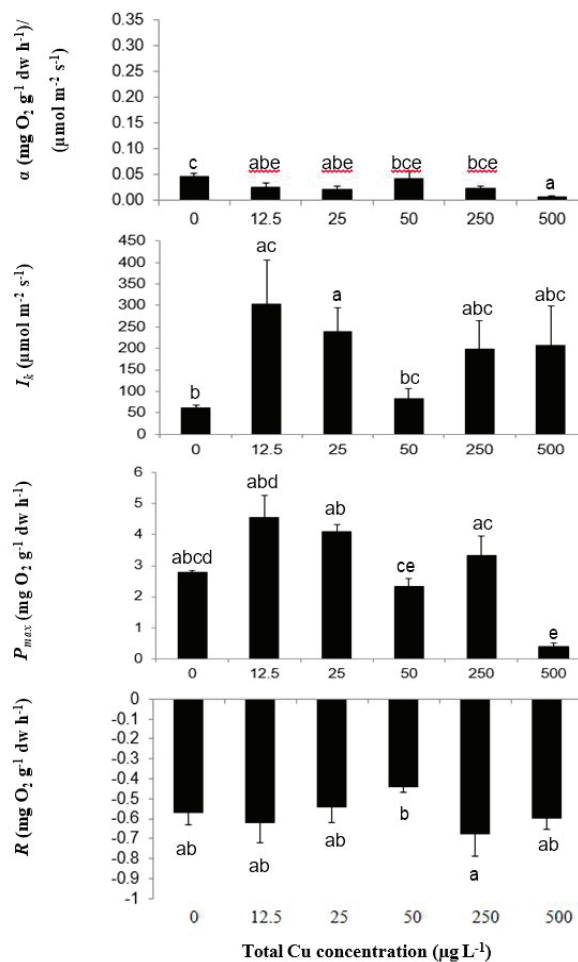

Figure 7. Phosynthesis-Irradiance parameters (mean±s.e.,*n*=6) of *Padina sanctae-crusis*. Two columns sharing the same letter are not significantly different.

to be bimodal. But statistically, significant differences were detected between 0 and 500 µg total Cu L^{-1}, 12.5 and 50, 12.5 and 500 µg total Cu L^{-1}, 25 and 50, 25 and 500 µg total Cu L^{-1}, and 250 to 500 µg total Cu L^{-1}. Photosynthesis for both species of algae was severely affected at the highest total Cu concentration.

The range of values for respiration rates between algae was comparable (Figures 6 and 7). For each alga, total Cu concentrations had a significant effect on respiration rate. Respiration rate of both algae exhibited a bimodal pattern with respect to total Cu concentrations. However, pairwise multiple comparisons displayed significant differences between 0 and 25, 12.5 and 25, 25 and 50, and 25 and 500 µg total Cu L^{-1} for *G. salicornia*, and between 50 and 250 µg total Cu L^{-1} for *P. sanctae-crusis*.

DISCUSSION

The relationship between WW and DW was undertaken in *P. sanctae-crusis* due to the observed wearing of the outer layer of the fronds which could lead to possible errors in computing net photosynthesis. The R^2 value of 0.96 was comparable to the findings of those who employed gravimetric procedures suggesting that for a definite WW, its DW can be determined. With such established WW-DW relationship, this would allow physiological studies related to the biomass of known individuals over time. On the other hand, the WW-DW relationship for *G. salicornia* was not done because intact thalli can still be collected after the P-I experiment.

Growth response to copper exposure and other observable changes
In the present study, EC_{50} was 80 µg total Cu L^{-1} and EC_{20} was 50 µg total Cu L^{-1} for *P. sanctae-crusis*. However,

only EC_{20} was evident at 100 µg total Cu L^{-1} for *G. salicornia* using the same range of total Cu concentrations. These results showed different sensitivity of algae across levels of total Cu concentrations. The significant interaction between algal type and total Cu concentrations further confirmed our observation on the growth rate of the two species. Variation among responses of algae to copper were found to be not related to cell size, cell wall type, taxonomic group, and K_d values (on a surface area basis) (Levy et al. 2008). The differences in sensitivity of algae may be due to the different mechanisms which are dependent on the nature of the pollutant and metabolic properties of algal species (Juneau et al. 2001) although it was not statistically shown in the P-I parameters except for P_{max}. This was corroborated by Levy et al. (2007) when they reported that the differences in sensitivity of algae may be due to differences in uptake rate across the plasma membrane, in internal binding mechanisms, and or detoxification mechanism between the different algal species.

Aside from the observed effects of copper on metabolic and physiological aspect of algae, a distinct yellowing of the medium was evident. Nielsen and Nielsen (2010) also observed the same event and confirmed it as polyphenols. Polyphenols are ubiquitous primary metabolites in brown algae that are suggested to have multiple transitional roles (Thomas 2001). It has strong *in vitro* antioxidant property owing to their ability to rapidly reduce reactive oxygen species (ROS) (Olivier 2012). Chlorosis symptoms (e.g., changes of the thalli color of *G. salicornia* and degradation of the outer layer of *P. sanctae-crusis*) were also observed. Such a color change following copper exposure was previously observed in brown algae due to the reduced chlorophyll and carotenoid levels (Nielsen and Nielsen 2010; Connan and Stengel 2011). However, Brown and Newman (2003) reported no corresponding reductions of chlorophyll and carotene concentration on red algae *Gracilariopsis longissima* although a decrease in phycobiliproteins was observed. Phycobiliproteins are photosynthetic energy transfer proteins that possess distinctively strong pigmentation and give the Rhodophyta the red color (Telford et al. 2001; Marsac 2003). Compared with chlorophyll-proteins, phycobiliproteins are more sensitive to perturbation by alterations to their chemical environment including oxiradicals which can be induced upon exposure to Cu (Brown and Newman 2003). On the other hand, the degradation of fronds of *P. sanctae-crusis* due to the elevated total Cu levels could be due to the disruption of photosynthetic units, disorganization of the chloroplast structure, lipid peroxidation, and breakage of the cells (Connan and Stengel 2011).

Comparison of P-I models

Several P-I models have been developed to express the relationship between photosynthetic rate and irradiance. Almost all models can estimate the initial slope (α), the light saturation (I_k), and the maximum production rate (P_{max}). The models have been applied to experimental data more or less successfully. An additional respiration term (R or R_d) was added to the end of the P-I equations to account for non-zero dark intercept values (Shafer et al. 2011). Nevertheless, these contemporary models are generally similar in shape with the previous P-I models.

In this study, two of the contemporary P-I models were optimized in fitting the data. Currently, there is no statistical significance test that identifies a correct model given the sample data, hence, it is necessary to take multiple criteria into consideration and to evaluate model fit on the basis of various measures simultaneously. For each estimation procedure, goodness of fit indices (R^2 and SSR) were provided to evaluate whether the model was consistent with the data. Aalderlink and Jovin (1997) compared eight different models and reported R^2 values from 0.31- 0.89 and grand SSR values of 2.71- 3.38 drawing a conclusion of no best fit model with respect to goodness of fit, as a result of having different biomass and light exposure among experiments. But, with proper experimental design, such experimental error can be mitigated. Hence, in this study Model 2 generated R^2 value of 0.95 and grand SSR value of 1.29, and Model 1 generated R^2 value of 0.94 and grand SSR value of 1.34. Based on this standard values, one can conclude that Model 2 fits the data slightly better than Model 1. However, calculating the goodness of fit alone for model selection is criticized by many, since such techniques neglects the principle of parsimony and consequently, can result in imprecise parameter estimates (Johnson and Omland 2004). In this study, the Akaike Information Criteria (Akaike 1981), a technique that considers both fit and complexity, was used to verify the selection of models. A smaller AIC_c indicates a better model fit. Model 2 generated smaller AIC_c values compared to Model 1. Thus, Model 2 was used to derive all P-I parameters.

Assessment of P-I curve parameters

Earlier studies of Cu^{2+} toxicity with ecologically plausible concentrations of Cu^{2+} reported that Cu^{2+} usually does not affect α (Ouzounidou et al. 1997; Ouzounidou 1996; Lidon et al. 1993). However, the results are different under high-irradiance conditions or long light phases. This was first found in the leaves of higher plants *Triticum aestivum* where Cu^{2+} toxicity caused a considerable decline of α (Lanaras et al. 1993). This was later followed by extensive studies of red and green algae (Küpper et al. 1998, 2002, 2003). Similar decreasing effects on α of both *G. salicornia* and *P. sanctae-crusis* were observed

in this study. It is often argued that copper-induced decrease of α is a consequence of inhibition of electron donation to the primary photochemical reaction (Küpper et al. 2002). Cu^{2+} treatment might, however, induce a state analogous to photoinhibition in which trapping of an exciton in the photosystem II (PSII) reaction center (RC) is followed by its nonradiative dissipation (Prasil et al. 1996). In the case of Cu^{2+}-induced inhibition, this state could most probably result from insertion of Cu^{2+} into the pheophytin *a* (Pheo *a*) molecule of the PSII RC. Pheophytin *a* is a primary electron donor in PSII of oxygenic photosynthetic organisms. Yruela et al. (1996) provide convincing evidence that the inhibition of PSII activity by Cu^{2+} occurs at the Pheo-Q_A domain which interferes with the stabilized separation. However, Yruela et al. (1993) concluded that the primary charge separation (i.e. the formation of the radical pair P680$^+$ Pheo$^-$) was not obstructed because of the accumulation of Pheo$^-$ in the presence of Cu^{2+} concentrations that inhibit the Q_A reduction. This was contested by Küpper et al. (2002) saying that the accumulation of Pheo$^-$ can be observed only in the presence of dithionite which will reduce cupric to cuprous and are likely to be inserted into Pheo$^-$. On the other hand, the findings of Yruela et al. (1993) that Cu^{2+} compete with H^+ for the inhibitory binding sites in PSII is actually in agreement with the idea proposed by Küpper et al. (2009) that heavy metals become bound to Pheo molecule, inactivating the PSII, and affecting its photochemical efficiency.

The most revealing total Cu-induced change in P-I parameters in this study were the increase or comparable degree of I_k values against the control of both algae. I_k is mostly dependent on the ratio of functional antenna molecules to functional reaction centers and electron transport chains (Mijovilovich et al. 2009). Under long light phase, high amount of photons captured by functional antenna molecules and delivered to its reaction center will cause more of an "electron traffic jam". These changes indicate a reduced ratio of functional PSII RCs to light harvesting complex II (LHCII) in a system with high antenna connectivity (Küpper et al. 2002) which is in line with reduced values of α that indicates damage of PSII RCs. If this state continues, I_k will appear to have a linear pattern against a certain variable. However, transient shifts on I_k at 50 µg L^{-1} for both algae were observed, producing another peak value. Morgan-Kiss et al. (2006) opined that the adjustment of the aforementioned ratios and the alteration of the photosynthetic unit to establish a long term acclimation to stressor could possibly explain the shift of the I_k. From the increasing I_k values observed on *G. salicornia* and *P. sanctae-crusis* against the control, it can be concluded that both algae can adapt to high irradiances. Plants adapted to higher irradiances usually have high capacity for photosynthesis (hence, their higher P_{max})

and contain more photosynthetic enzymes which require Cu^{2+} ions. These sun-adapted plants can accumulate high concentration of Cu^{2+}-requiring photosynthetic enzyme plastoquinone in the lipid reservoir of the chloroplast stroma (Lichtenthaler 2007). Furthermore, White et al. (1996) observed an increase of ROS such as superoxide dismutase that also contains Cu^{2+} in response to high irradiance. These Cu^{2+}-requiring enzymes may act as extra sinks to total Cu. The higher total Cu requirement is clearly expressed by *P. sanctae-crusis*. P_{max} of *P. sanctae-crusis* is higher in algae exposed to 12.5- 25 µg total Cu L^{-1} compared to the controls. The Cu^{2+} requirement as well as the tendency of higher plants to exclude Cu^{2+} (Lehotai et al. 2011) may lead to copper deficiency that may also lead to suboptimal level of plastoquinone and superoxide dismutase on algae exposed to Cu^{2+}-free environment. Nielsen and Nielsen (2010) reported a non-photochemical quenching (NPQ) in the absence of Cu^{2+}. Non-photochemical quenching (NPQ) is normally associated with dynamic photoinhibition and xantophyll cycle-dependent energy dissipation in macroalgae and higher plants (Muller et al. 2001). Therefore, the lower P_{max} in the control compared to algae exposed to 12.5- 25 µg total Cu L^{-1} is not a result of photodamage, but is hypothesized to be a result of total Cu limitation. Unlike *P. sanctae-crusis*, *G. salicornia* exposed to the control medium as well as those at 12.5- 25 µg total Cu L^{-1} were comparable. This may be because of its differences in Cu requirement or may be due to the higher thallus surface area of *P. sanctae-crusis* compared to *G. salicornia,* possibly allowing a higher and easier absorption (Connan and Stengel 2011) of copper by *P. sanctae-crusis*. But, both algae showed decreasing P_{max} values starting from 50 to 500 µg total Cu L^{-1} suggesting the detrimental effects of total Cu at higher concentration. Excess Cu^{2+} can lead to a reduction of PSII efficiency and is reportedly found to coincide with increased non-photochemical quenching (NPQ) (Nielsen and Nielsen 2010). Excitation energy trapped within PSII in thalli of *G. salicornia* and fronds of *P. sanctae-crusis* exposed to excess total Cu appears to be dissipated through quenching mechanisms similar to relieving light-induced photoinhibition. Failure to disperse or relieve excess supply of electrons in response to inhibition of electron transport may reflect a smaller robust pool of xantophylls (Nielsen et al. 2003). Moreover, excess excitation energy can induce production of ROS leading to disruption of photosynthetic units and chlorophyll breakdown (Muller et al. 2001).

Respiration rate, represented by oxygen consumption, varied with different total Cu concentrations producing a non-linear pattern. Pasichnaya (2002) reported an augmented O_2 intake resulting from an increased Cu^{2+} concentration in the medium, which is accordingly connected with a necessity to compensate a deficiency

of energy resources. Cid et al. (1995) also concluded that any compound which affects photosynthesis would also be expected to alter the ATP intracellular content. It is important to note that the chloroplast exports high energy molecules to the cytoplasm, but all the ATP needed in the cytoplasm comes from the mitochondria (Heldt 2002). From this point of view, the higher respiration rate at intermediate total Cu concentrations (i.e., 25 µg total Cu L^{-1} for *G. salicornia* and 250 µg total Cu L^{-1} for *P. sanctae-crusis*) in this study can be a consequence of an increased energy demand for active transport, production of specific metal-binding polypeptides, removal of the resulting metal-phytochelatin from the cytoplasm to the vacuole and organelles, production of extracellular compound, and the synthesis of enzymes and compounds related to oxidative stress (Perales-Vela et al. 2007). However, this does not explain why the *R* values are higher at intermediate total Cu concentration. The absence of a non-linear pattern in *R* with total Cu concentration could be due to delayed effect of copper on the respiratory system.

CONCLUSION

To sum it up, the results of this study strongly suggest severe impact of total Cu at high concentrations on P-I parameters and growth rate of *G. salicornia* and *P. sanctae-crusis*.

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