Short-term effects of heavy metal and temperature stresses on the photo-physiology of *Symbiodinium* isolated from the coral *Fungia repanda*

MANJULA D. GHOORA¹, SIVAJYODEE S. PILLY¹, PRAMOD KUMAR CHUMUN², SHOBHA JAWAHEER^{1,♥},
 RANJEET BHAGOOI^{1,2,3,4,♥♥}

¹Department of Biosciences and Ocean Studies, Faculty of Science, University of Mauritius. Réduit 80837, Republic of Mauritius.

♥email: shobha@uom.ac.mu

²The Biodiversity and Environment Institute. Réduit, Republic of Mauritius. Tel.: +230-4037916, Fax.: +230-4656928, ♥♥email: r.bhagooli@uom.ac.mu

³Pole of Research Excellence, Sustainable Marine Biodiversity Research Group, University of Mauritius. Réduit 80837, Republic of Mauritius

⁴Institute of Oceanography and Environment (INOS), Universiti Malaysia Terengganu. 21030 Kuala Terengganu, Terengganu, Malaysia

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Abstract. Ghoola MD, Pilly SS, Chumun PK, Jawaheer S, Bhagooli R. 2018. Short-term effects of heavy metal and temperature stresses on the photo-physiology of *Symbiodinium* isolated from the coral *Fungia repanda*. *Ocean Life* 2: 11-20. This study aimed to investigate the effects of the heavy metals, copper, zinc and lead, on the photo-physiology of the symbiotic dinoflagellate *Symbiodinium* isolated from the coral *Fungia repanda*. Freshly isolated *Symbiodinium* found to belong to clade C were exposed to different concentrations of the three heavy metals for 3-hour and 18-hour treatments at 28°C and 32°C. The Pulse Amplitude Modulated (PAM) fluorometry technique was used to determine the maximum quantum yield (F_v/F_m), relative maximum electron transport rate ($rETR_{max}$) and maximum non-photochemical quenching (NPQ_{max}) of the photosystem II (PSII). An increase in non-photochemical quenching accompanied by a decrease in photosynthetic capacity was noted for copper at a concentration of 50 µg/L for both temperatures. The F_v/F_m was not significantly affected by the Zn treatments. However, at 28 °C, isolates treated with 100 µg/L Zn for 18 hours showed an increase in non-photochemical quenching accompanied by a decrease in photosynthetic capacity. Pb had the most profound effect on all of the isolates. The F_v/F_m significantly decreased and an increase in NPQ_{max} was noted. The decrease of $rETR_{max}$ and increase in NPQ_{max} for the heavy metal bioassays under 32 °C were more significant than at 28 °C. This study suggests that Cu (≥ 50 µg/L), Zn (≥ 100 µg/L) and Pb decrease the photosynthetic capacity of the *Symbiodinium* isolates from *F. repanda* especially more so with increasing temperatures.

Keywords: *Fungia*, heavy metal, photosynthetic parameters, Pulse Amplitude Modulated fluorometry, *Symbiodinium*, thermal stress

Abbreviations: PAM: Pulse Amplitude Modulated; F_v/F_m : maximum quantum yield; $rETR_{max}$: relative maximum electron transport rate; NPQ_{max} : maximum non-photochemical quenching; PSII: photosystem II; rpm: revolutions per minute; RLCs: rapid light curves; F_o : initial fluorescence; F_m : maximum fluorescence

INTRODUCTION

Although rising seawater temperature, one of the major indicators of global climate change (National Climatic Data Centre 2011), might exert damaging effects on the marine biota in the long term (from decades to centuries), chemical contaminants such as heavy metal pollution may pose more immediate threats to the coastal residents (Hu et al. 2017). Release of heavy metals to the marine environment mainly results from atmospheric and river inputs, direct discharges, industrial dumping and sewage sludge, among the important contributors to metal pollution (Valavanidis and Vlachogianni 2010). At low concentrations, heavy metals are essential to the metabolism of the organisms, but at higher levels they may lead to toxicity (Phillips 1995; Sunda and Huntsman 1998; Pinto et al. 2003). Heavy metals are known to reduce photosynthesis by affecting the light harvesting complex, oxygen evolution complex, cytochrome complex, plastoquinone, plastocyanin, ferredoxin and NADP⁺ (Baumann et al. 2009).

The marine environment undergoes rapid fluctuations in seawater temperature which may change the conditions necessary for optimum metabolism (Oukarroum et al.

2012). Field and laboratory studies on corals and their symbiotic associations have established a causal link between temperature stress and bleaching events (Lesser 1996) in symbiotic corals that build reefs. Exposure to sub-lethal temperatures (Iglesias-Prieto et al. 1992) leads to photoinhibition of photosynthetic processes in marine organisms. Elevated temperature has been found to cause damage to the photosystem II (Warner et al. 1999) and recovery of the D1 protein (Takahashi et al. 2009) which forms part of the water-splitting complex in photosystem II. Moreover, the Calvin-Benson cycle is compromised under high temperature exposures (Jones et al. 1998; Bhagooli and Yakovleva 2004; Bhagooli and Hidaka 2006) and the site of damage has been speculated to be the enzyme RuBisCO (Lesser 1996; Lilley et al. 2010). Temperature increase in aquatic systems has also been found to enhance the toxicity of some metals on algae (Cairns et al. 1975; Heugens et al. 2001) by increasing the rate of diffusion or active transport.

Environmental stresses pose a threat to the fragile coral reef ecosystems, which are hosts to a highly diverse group of dinoflagellate symbionts of the genus *Symbiodinium* (Baker 2003). These symbionts are responsible for the

existence of the coral reefs as we know them (Stanley and Swart 1995) and contribute substantially to coral reef productivity. This study focused on the scleractinian coral *Fungia*, which is a genus tolerant to environmental stresses (Mattan-Moorgawa et al. 2011). Many studies used the chlorophyll *a* fluorescence technique estimated by the pulse-amplitude-modulated (PAM) fluorometer to assess the photo-physiology of corals and/or their associated symbionts under heavy metal stress (Bielfmyer et al. 2010; Gorbunov and Falkowski 2011) or temperature stress (Bhagooli and Hidaka 2002, 2006; Bhagooli and Yakovleva 2004) as individual stress factors. A few studies even looked at the interactive effects of heavy metal and temperature on photosynthetic physiology (Baumann et al. 2009; Oukarroum et al. 2012) but Baumann et al. (2009) worked with macroalgae over a 14-day period and Oukarroum et al. (2012) worked with cultured microalgae over a 24-hour period. To the best of our knowledge, there is a dearth of information on the short-term effects of heavy metals assessed individually and in combination with temperature stress on the photosynthetic physiology of freshly-isolated symbionts of a thermally resistant coral-*Fungia repanda*. The main objective of the present study is thus to expose freshly isolated *Symbiodinium* of *F. repanda* to increasing concentrations of heavy metals namely Cu, Zn and Pb for 3-hour and 18-hour treatments under two temperatures-28°C and 32°C in order to assess the photosynthetic physiology of the organism in response to the stress conditions using PAM fluorometry to determine the three chlorophyll *a* fluorescence parameters-the maximum quantum yield (F_v/F_m) of PSII, the maximum relative electron transport rate ($rETR_{max}$) and the maximum non-photochemical quenching (NPQ_{max}).

MATERIALS AND METHODS

Specimen collection and symbiont isolation

Medium-sized scleractinian coral individuals of *Fungia repanda* (diameter ~10 cm) were collected at a depth of ~ 2 m at Trou aux Biches, one of the world-renowned beaches located on the northern coast of the island of Mauritius (20.0350 °S, 57.5450 °E) (Figure 1). The reefs of Trou aux Biches harbor a diversity of coral species including *Acropora*, *Alveopora*, *Echinopora*, *Favia*, *Favites*, *Fungia*, *Galaxea*, *Pavona*, *Pocillopora*, *Porites* amongst various other genera (AIMS 2017).

The coral was allowed to recover from handling in a plastic container filled with seawater for 1 hour at ambient temperature (25.0 ± 1.0 °C) prior to further processing. *Symbiodinium* cells were obtained from *F. repanda* by blasting the coral with filtered sea water (FSW) (0.47 µm) using an oral hygiene device (Water Pik). The blasted tissue was then homogenized using a tissue grinder at 9500 rpm. The homogenate was filtered twice, first with a coarse (180 µm) and then a fine (35 µm) filter mesh and subsequently centrifuged at 2000 x g for five minutes. The pellets were re-suspended with FSW before a second centrifugation at 1800 x g for ten minutes to obtain clean *Symbiodinium* pellets. A cell count was performed using the Neubauer Hemocytometer Chamber and 1 ml of the isolated symbionts suspension was adsorbed onto 0.22 µm Millipore filters (Ø-13 mm) using a syringe apparatus. Cell densities of above 10^5 cells cm^{-2} were used for experimental trials as these densities ensure reliable PAM measurements (Bhagooli and Hidaka 2004a).

Figure 1. Mauritius and its aerial view showing the sampling site, Trou aux Biches

Experimental protocol

In the laboratory, *Symbiodinium* cells were harvested on millipore filters and separately exposed to four different concentrations of the heavy metals Cu, Zn and Pb. For each heavy metal assay, the *Symbiodinium* was cultured under two temperature regimes, 28 °C and 32 °C, and two exposure period, 3-h and 18-h. Each test was carried out in triplicate. These two temperatures were chosen to represent two conditions leading to non-bleaching and bleaching responses, respectively, in corals reported from Mauritian waters (Bhagooli and Taleb-Hossenkhani 2012; Mattanmoorgawa et al. 2012) and the Great Barrier Reef (Jones et al. 1998). Heavy metal test concentrations were prepared by dilution of standard solutions of Cu, Zn and Pb (1000 ppm) with sea water filtered through a 0.2 µm membrane filter (Schleicher and Schuell Nitrocellulose Membrane Filters) to produce the following concentrations-Cu: 0, 10, 30 and 50 µg/L; Zn: 0, 25, 50 and 100 µg/L; Pb: 0, 10, 30 and 50 µg/L. The concentrations were chosen based on the range of levels of heavy metals reported in coral reefs areas (e.g. Ali et al. 2011) and set toxic thresholds (ANZECC 1992). Five millilitres of each heavy metal solution was added to McCartney bottles followed by subsequent addition of 1 Millipore filter with adsorbed symbionts per vial. The McCartney bottles were then immersed in two waterbaths, one set at 28 °C and the other one at 32 °C. The treatments were illuminated by a light source of 200 µmol m⁻² s⁻¹ measured by a light meter (Hagner Digital Luxmeter, EC1-Y) during the 3-h and 18-h stress.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured using a Pulse Amplitude Modulated (PAM) fluorometer (DIVING-PAM, Heinz Waltz GmbH, Germany). The initial fluorescence (F_o) was determined by applying a weak pulse-modulated measuring light (< 0.1 µmol quanta m⁻² s⁻¹) when the PSII reaction centres are open. The maximum fluorescence (F_m) was determined after applying a saturating pulse (> 6000 µmol quanta m⁻² s⁻¹) when the reaction centres are closed. The ratio of the change in fluorescence to maximum fluorescence ($[F_m - F_o] / F_m$) gives the dark-adapted photosynthetic parameter F_v/F_m , which is a good proxy of the maximum quantum yield of PSII (Genty et al. 1989). Samples were dark-adapted for 15 minutes prior to measurement. After the dark-adapted measurement, the samples were light adapted for 5 minutes, followed by 30 s dark period, and initial and maximum fluorescence (F_o' and F_m' respectively) were determined again. The product of the ratio of change in fluorescence to maximum fluorescence of light-adapted samples ($[F_m' - F_o'] / F_m'$) (also known as the effective quantum yield) and the photosynthetically active radiation (PAR) gives the parameter relative Electron Transport Rate ($rETR$). The non-photochemical quenching (NPQ) parameter, which regulates dissipation of excess energy in the form of heat, is derived from the ratio of change in maximum fluorescence from the dark-adapted to the light-adapted stage, to the maximum fluorescence of the illuminated

sample ($NPQ = [F_m - F_m'] / F_m'$) (Bilger and Björkmann 1990).

The $rETR$ and the NPQ were derived from the rapid light curves (RLCs) obtained after light adapting the samples. The RLCs determines the physiological flexibility of the symbionts to adapt their photosynthetic apparatus to rapidly changing light intensities. Rapid irradiances occurred at an interval of every 10 s and gave fluorescence measurements which were fitted as an exponential decay curve. The $rETR_{max}$ was obtained by fitting $rETR$ curves in the Sigma Plot software using the Platt et al. (1980) equation. NPQ_{max} represents the highest non-photochemical quenching value.

Symbiodinium isolation, DNA extraction and clade identification

Coral tissues were removed using a waterpik and filtered seawater (FS, 0.45µm). The blastate was centrifuged for 10min at 4000 rpm, washed with filtered seawater and centrifuged again to pellet the *Symbiodinium* cells. The pellet was suspended in 1ml of FS. Following centrifugation for 5 min at 4000 rpm the pellet was resuspended with 1% sodium dodecyl sulfate (SDS) and DNA isolation buffer (0.4 M NaCl; 50 mM EDTA, pH 8), vortexed, treated for 1-2 hrs at 65°C and stored at room temperature for later analyses. DNA extraction, was carried out using slightly modified method of Rowan and Powers (1991). Proteinase-K was added to the *Symbiodinium* suspension and incubated for 2-3 hrs at 55°C. 64 µl of 5M NaCl was added followed by 60 µl of 10% cetyltrimethylammonium bromide (CTAB) and was topped up to 600µl with sterile distilled water. The lysate was then heated for 30min at 65°C followed by addition of 600µl of chloroform. The lysate was subject to chloroform extraction once and phenol extraction twice. 900µl of cold ethanol was then added followed by 45µl 3M Sodium Acetate (NaOAc). The DNA was precipitation at -20°C overnight and excess chloroform was washed with 70% ethanol. The DNA was then air dried and re-suspended in 50µl TE buffer. Polymerase chain reaction (PCR) was done using *Symbiodinium* specific primers ss3z and ss5z that anneal to the 18S-rDNA region of the *Symbiodinium* DNA. Restriction digest was performed by incubating the PCR product for 2 hours with Taq I enzyme. The banding pattern of the RFLP was then visualize in agarose gel.

Statistical analyses

Chlorophyll fluorescence data was arcsine transformed prior to statistical analyses. Multivariate analysis of variance (ANOVA) was carried out using the statistical software STATISTICA version 10.0 to compare the effects of the heavy metals (Cu, Zn and Pb) and their respective concentrations, temperatures (28°C and 32°C) and exposure times (3-h and 18-h) per se and in combination on the photosynthetic parameters, F_v/F_m , $rETR_{max}$ and NPQ_{max} . Differences between groups were determined by the Post Hoc Tukey HSD test.

RESULTS AND DISCUSSION

Genotyping results showed that *F. repanda* harboured the *Symbiodinium* Clade C (Figure 2). An increase in temperature, heavy metal concentrations, and exposure time reduced the maximum quantum yield (F_v/F_m) of the symbionts significantly ($P < 0.05$). The maximum non-photochemical quenching (NPQ_{max}) was increased significantly ($P < 0.001$) by temperature, heavy metal concentration and exposure time. However, no marked difference ($P > 0.05$) was noted across the three heavy metals. The $rETR_{max}$ remained invariant ($P > 0.05$) under temperature stress but was significantly reduced by the heavy metals and their concentrations and exposure time. Interaction of stress factors, evaluated by the multivariate ANOVA analyses had variable effects on the photosynthetic parameters as shown in Table 1.

Effects of Cu

Figure 3 shows the variation of the three fluorescence-based parameters, F_v/F_m , $rETR_{max}$ and NPQ_{max} with Cu concentrations over three and eighteen-hour treatments. Zooxanthellae isolated from *F. repanda* had initial F_v/F_m values of 0.593 ± 0.011 . At 28 °C, no change in F_v/F_m was noted ($P > 0.05$), the light-adapted parameters were affected significantly; 50 µg/L copper reduced the $rETR_{max}$ significantly for all isolates ($P < 0.001$ for 3 h; $P < 0.01$ for 18 h) and increased the NPQ_{max} for the 18 h treatment ($P < 0.01$). The combined effects of copper and high temperature stresses (32°C) considerably reduced the photosynthetic outputs of PSII. Although 10µg/L copper did not affect the photosynthetic parameters measured, a significant reduction in $rETR_{max}$ ($P < 0.001$) during the 18 h treatment was evident with 30 µg/L Cu. The highest copper concentration used (50 µg/L) caused marked decreased in F_v/F_m ($P < 0.05$ for 3 h; $P < 0.01$ for 18 h) and $rETR_{max}$ ($P <$

0.01 for 18 h; no change for 3 h) and significant increase in NPQ_{max} ($P < 0.001$ for both 3h and 18 h).

Effects of Zn

Zn exposure on isolated *Symbiodinium* (Figure 4) showed no significant change in F_v/F_m for all treatments. However, the $rETR_{max}$ was significantly reduced at 28°C when the symbionts were treated with 100 µg/L zinc for 18 h ($P < 0.05$). An associated increase in NPQ_{max} ($P < 0.01$) was noted for the same treatment. At 32°C, zinc concentrations of 50µg/L and 100µg/L caused significant increase in NPQ_{max} ($P < 0.001$ for 50 µg/L; $P < 0.01$ for 100 µg/L) but not the other measured photosynthetic parameters.

Figure 2. Restriction fragment length polymorphism (RFLP) of *Symbiodinium* Clade C harbored by *F. repanda*. C-clade C; L-DNA ladder; FUN-*F. repanda*

Table 1. Summary of multivariate ANOVA analyses testing the effect of temperature (28°C and 32°C), heavy metals (Cu, Zn and Pb) and their respective concentrations (Cu and Pb: 0, 10, 30 and 50 µg/L; Zn: 0, 25, 50 and 100 µg/L) and exposure time (3h and 18h) individually and in combination (s), on F_v/F_m , NPQ_{max} and $rETR_{max}$. Significant differences are indicated in red. [Abbreviations: Temp: temperature; HM: heavy metals; Conc: concentration; DF: degree of freedom; MS: mean square; F: variance ratio; P: probability value] (n = 3)

Source of variation	F_v/F_m				NPQ_{max}				$rETR_{max}$			
	DF	MS	F	P	DF	MS	F	P	DF	MS	F	P
Temp	1	0.001	4	0.037	1	0.018	29.66	0.000	1	0.004	3.70	0.057
HM	2	0.001	9	0.000	2	0.001	1.08	0.341	2	0.004	4.35	0.015
Conc	3	0.005	35	0.000	3	0.038	63.76	0.000	3	0.027	25.88	0.000
Time	2	0.005	36	0.000	2	0.039	65.51	0.000	2	0.223	217.36	0.000
Temp*HM	2	0.000	0	0.890	2	0.003	5.59	0.005	2	0.008	7.33	0.001
Temp*Conc	3	0.000	1	0.512	3	0.001	1.47	0.226	3	0.001	1.20	0.312
HM*Conc	6	0.000	1	0.536	6	0.000	0.68	0.663	6	0.002	1.68	0.131
Temp*Time	2	0.000	2	0.120	2	0.005	7.93	0.001	2	0.007	6.88	0.001
HM*Time	4	0.000	2	0.047	4	0.002	3.93	0.005	4	0.008	7.46	0.000
Conc*Time	6	0.000	2	0.172	6	0.011	18.98	0.000	6	0.009	8.69	0.000
Temp*HM*Conc	6	0.000	0	0.933	6	0.000	0.71	0.640	6	0.005	5.36	0.000
Temp*HM*Time	4	0.000	0	0.899	4	0.002	2.65	0.036	4	0.008	7.67	0.000
Temp*Conc*Time	6	0.000	0	0.950	6	0.001	2.16	0.050	6	0.002	1.92	0.081
HM*Conc*Time	12	0.000	0	0.993	12	0.001	2.45	0.006	12	0.003	2.55	0.004
Temp*HM*Conc*Time	12	0.000	0	0.998	12	0.001	1.39	0.179	12	0.004	3.55	0.000

Figure 3. Maximum quantum yield of PSII (F_v/F_m), maximum relative electron transport rate ($rETR_{max}$) and non-photochemical quenching (NPQ_{max}) of zooxanthellae isolated from *F. repanda* under Cu treatments at 28°C (A, B and C) and 32°C (D, E and F). Note: * represents $P < 0.05$, ** represents $P < 0.01$ and *** represents $P < 0.001$ between the treatment and initial. Data are represented as mean $\pm$ standard deviation ($n = 3$).

Figure 4. Maximum quantum yield of PSII (F_v/F_m), maximum relative electron transport rate ($rETR_{max}$) and non-photochemical quenching (NPQ_{max}) of zooxanthellae isolated from *F. repanda* under Zn treatments at 28°C (A, B and C) and 32°C (D, E and F). Note: * represents $P < 0.05$, ** represents $P < 0.01$ and *** represents $P < 0.001$ between the treatment and initial. Data are represented as mean $\pm$ standard deviation ($n = 3$).

Figure 5. Maximum quantum yield of PSII (F_v/F_m), maximum relative electron transport rate ($rETR_{max}$) and non-photochemical quenching (NPQ_{max}) of zooxanthellae isolated from *F. repanda* under Pb treatments at 28°C (A, B and C) and 32° (D, E and F). Note: * represents $P < 0.05$, ** represents $P < 0.01$ and *** represents $P < 0.001$ between the treatment and initial. Data are represented as mean $\pm$ standard deviation ($n = 3$)

Effects of Pb

Pb reduced the photosynthetic capacity of the isolated *Symbiodinium* by affecting all the photosynthetic parameters monitored (Figure 5). A pronounced decrease was noted in F_v/F_m over both temperature treatments (28 °C and 32 °C). While the F_v/F_m value of the 50µg/L lead treatment over 3 h was significantly reduced ($P < 0.05$) at 28°C, marked reduction was noted as from 30µg/L for the same parameter at 32 °C; the symbionts exposed for 3 h and 18 h under 30 µg/L and 50 µg/L lead treatments at 32°C had significantly lower F_v/F_m values (30 µg/L: $P < 0.001$ for 3 h, $P < 0.05$ for 18 h; 50 µg/L: $P < 0.001$ for 3 h, $P < 0.01$ for 18 h). The $rETR_{max}$ was significantly reduced after 18 h at lead concentrations of 30µg/L and 50µg/L for the 28 °C treatment ($P < 0.001$) but not for 32 °C. However, for treatments at both temperatures (28 °C and 32 °C) showed a significant increase in NPQ_{max} ($P < 0.001$ for 3 h at 28 °C, $P < 0.001$ for 18 h at 32 °C).

Discussion

A Cu concentration of 50 µg/L did not result in any significant changes in F_v/F_m , however, a decrease in $rETR_{max}$ accompanied by an increase in NPQ_{max} was recorded within 18hrs of exposure. This suggests that 50 µg/L Cu decreased the photosynthetic capacity of the *Symbiodinium* and the excess energy was effectively dissipated. This is consistent with Yruela et al. (1992) who noted that Cu inhibits electron transfer at the level of pheophytin Q_A -Fe domain of the PSII reaction centre and

Han et al. (2008) who noted no significant change in photosynthetic yield at Cu concentrations 25-50 µg/L but a higher Cu concentration of 250 µg/L did reduce the F_v/F_m significantly. A large body of research has shown that Cu is toxic to the photophysiology of marine organisms and cause damage to several target sites along the photosynthetic pathway (Parales-Vela 2007; Han et al. 2008; Bielmyer et al. 2010; Connan and Stengel 2011; Oukarroum et al. 2012). As toxicity is generally considered to be dose-dependent, a high Cu concentration is expected to cause decline in the photosynthetic yield. Moreover, toxicity is linked to the sensitivity of the test organisms since Cu concentration as low as 4 µg/L has been found to reduce the quantum yield in algal symbionts of *Pocillopora damicornis* as reported by Bielmyer et al. (2010). Kuzminov et al. (2013) investigated Cu toxicity over several days in a cultured *Symbiodinium* (CCMP 2467) isolated from the coral *Stylophora pistillata*. They reported no significant change in F_v/F_m and slight but not significant increase in maximum rate of photosynthetic electron transport (P_{max}) up to 2 days of exposure to 50µM Cu at 25°C. However, after 3 days exposure, was observed significant decline in both F_v/F_m and P_{max} . They also reported that the time of electron transport between photosystems ($\tau_{PSII-PSI}$) increased significantly within 12hrs of treatment. It is noteworthy that in higher plants such as *Arabidopsis thaliana*, a significant increase in ETR after exposure to Cu concentrations of 50-100 µg/L was observed (Martínez-Peñalver et al. 2012) suggesting that

Cu which is a micronutrient may have been limiting in the multicellular organism.

A significant reduction in the $rETR_{max}$ was recorded within 18hrs of exposure to Zn which is in line with other investigations on the effect of Zn on photosynthesis carried out by many authors (Davies and Sleep 1979; Tripathy and Mohanty 1980; El-Sheekh 1993). Experiment using the O_2 -evolution method has shown that Zn inhibits the photosynthetic electron transport through PSII (Tripathy and Mohanty 1980) and this corresponds with the results of the present study where Zn has been shown to exert its effects at the oxidizing (H_2O -splitting) side of PSII, possibly inhibiting the manganese complex (Miller and Cox 1983; Van Assche and Clijsters 1986). Baker et al. (1982) proposed a second site for Zn^{2+} action in the electron transfer chain between the PSII and the PSI and this has been attributed to plastoquinone (Mohanty et al. 1989). This is in line with the observed decrease in $rETR_{max}$. Though the F_v/F_m did not vary significantly, it has been proposed that F_v/F_m is not sensitive to Zn and hence it may not be a good indicator of Zn stress (Joshi and Mohanty 2004). Baumann et al. (2009) reported significant reduction in yield of macroalgae when exposed to Zn concentration of 10 $\mu g/L$ after 4 days. In the latter study, Zn was reported to irreversibly bind to the test macroalgal species, causing death of the organisms which was confirmed by F_v/F_m values of zero. Kuzminov et al. (2013) reported Zn toxicity in a cultured *Symbiodinium* and found no significant change in F_v/F_m and but significant increase in maximum rate of photosynthetic electron transport (P_{max}) up to 2 days of exposure to 100 μM Zn at 25°C. After 3 days exposure significant decline in both F_v/F_m and P_{max} was observed.

Studies have reported that Pb stress can cause inhibition of photosynthesis at the level of the light harvesting complexes of PSI and PSII (Miles et al. 1972) and photosynthetic reduction cycle (Stiborova et al. 1986). Moreover, PSII has been found to be more sensitive to Pb than PSI. Pb inhibition site is located at the donor side of PSII, between the oxygen-evolving complex and the reaction centre of PSII (Joshi and Mohanty 2004). This is in accordance with a decrease in $rETR_{max}$ with increasing concentration of Pb. While in the present study F_v/F_m of *Symbiodinium* was severely reduced when exposed to a Pb concentration of 50 $\mu g/L$, Baumann et al. (2009) reported Pb to be one of the least toxic among 5 metals including Cu and Zn. In the latter study, Pb caused no reduction in fluorescence yield of 7 species of macroalgae at 10 $\mu g/L$ possibly because the macroalgae were tolerant to moderately high Pb concentration (Strömberg 1980; Lamai et al. 2005). However, Hussain et al. (2006) found drastic reduction in yield parameters when marsh plants were exposed to 20-40 mg/L Pb. Reduction in photosynthesis in algae by Pb has also been reported by Woolery and Lewin (1976). Extensive inhibition of photosynthetic electron transport was observed when isolated chloroplasts were exposed to 2.4 mM Pb for a few minutes (Miles et al. 1972) and this corresponds to the effects of Pb in our study. As a result, the system significantly increased its non-photochemical quenching to safely harness the excitation

energy. Kuzminov et al. (2013) documented Pb toxicity in a cultured *Symbiodinium* and reported no significant change in F_v/F_m up to 3 days of exposure to 50 μM Pb at 25°C but slight decrease in maximum rate of photosynthetic electron transport (P_{max}) up to 2 days of exposure. After 4 days exposure a significant decline in both F_v/F_m and P_{max} was observed. However, $\tau_{PSII-PSI}$ was the first parameter to be affected.

Under stress conditions such as combined heavy metal and thermal stress, the higher capacity for non-photochemical quenching helps to provide protection to the photosynthetic organism. In the present study, all heavy metal treatments carried out at the elevated temperature (32°C) recorded significantly high NPQ_{max} . A proposed photoprotection mechanism involves the inter-conversion between the two pigments diatoxanthin and diadinoxanthin (Ting and Owens 1993). Ruban et al. (2004) demonstrated that NPQ is tightly correlated to the presence of diatoxanthin and that the triggering key factor was the proton gradient across the thylakoid membranes. It is likely the alternative sources of protons such as the PS I cyclic electron transfer and/or chlororespiration are important in generating the proton gradient sufficient to trigger NPQ . Excess energy dissipation in the form of heat prevents the formation of reactive oxygen species which can induce lipid peroxidation and destruction of membrane structure and function. Both excess essential and non-essential metals, and elevated temperature are known to affect algal and coral, among other coastal species, physiology, metabolism and growth (El-Sarraf and Taha 1995; Bertrand and Poirier 2005; Mitchelmore et al. 2007; Baumann et al. 2009; Bielmyer et al. 2010; Main et al. 2010; Connan and Stengel 2011; Kuzminov et al. 2013). It is noteworthy that along with heavy metal stresses, the combined effects of temperature pose a greater threat on marine life forms (Cairns et al. 1978; Sokolova and Lannig 2008; Oukarroum et al. 2012). As noted by Oukarroum et al. (2012), heavy metal toxicity on photosynthetic performance is temperature-dependent, consistent with the present study. However, research carried out by Cairns et al. (1978) on four algal species revealed differential effects of heavy metal toxicity to temperature most probably due to different culturing methods of the algae, representing distinctly different habitats. The Mauritian waters is not spared from both metal contamination (Daby 2006) and elevated thermal anomalies (Mattan-Moorgawa et al. 2012; Bhagooli and Taleb-Hossienkhan 2012; Bhagooli and Sheppard 2012).

Time of stress under heavy metals significantly influenced the photosynthetic parameters in this study. Algal cells can accumulate heavy metals when exposed at high concentrations and these heavy metals can interfere with photosynthesis. However, specific responses of a given heavy metal on photosynthesis vary among species, thus broad generalization cannot be made about the combined effects of heavy metal exposure and time. The severity of the stress response depends on the exposure time as well as the concentration of heavy metals. Mitchelmore et al. (2007) showed that the coral *Pocillopora damicornis* could accumulate Cu 3-fold and

30-fold at 5 and 50 $\mu\text{g l}^{-1}$, respectively, after 4d of exposure, with the *in hospite Symbiodinium* accumulating 1.5-fold of Cu in 5 $\mu\text{g l}^{-1}$ treatment higher than that in the control. Bielmyer et al. (2010) investigated the effect of exposure of Cu on the coral *A. cervicornis* for 5 weeks and observed that Cu exposure and accumulation may affect the symbiont by reducing CO_2 available for photosynthesis. Kuzminov et al. (2013) also reported exposure time-dependent toxicity of essential and non-essential metals along with differential photophysiological responses to the metals of cultured *Symbiodinium*. However, sensitivity to heavy metals may vary with the organism's physiology and hence, it is important to understand the mechanisms of action of these heavy metals to better evaluate the effects of heavy metal stress. It is also noteworthy that the accumulation of heavy metals for an effective concentration resulting in significant photophysiological changes may be time-dependent and thus short-term exposures in hours may need to be extended to days of exposures to be able to thoroughly evaluate impacts of heavy metals on *Symbiodinium*.

Rise in the surface sea water temperature is expected to cause mass bleaching events leading to 'extinction' of some coral reefs in Mauritius and the 'extinction dates' have been suggested to occur between the years 2025-2070 based on the bleaching/mortality thermal threshold (Bhagooli and Sheppard 2012). This situation is further aggravated in the presence of heavy metal contaminants. Sokolova and Lannig (2008) reported synergistic effects of temperature and heavy metal stress. Elevated temperature is known to increase the rate of uptake and accumulation of heavy metals (Cairns et al. 1975; McLusky et al. 1986; Hutchings et al. 1996; Heugens et al. 2002). *Symbiodinium* exposed to thermal stress demonstrated a reduction in the dark-acclimated maximum quantum yield of PSII compared to the non-stressed ones (Hoegh-Guldberg 2005). In the present study, Cu and Pb treatments at 32°C significantly reduced the F_v/F_m , suggesting damage at the level of photosynthetic functioning in *Symbiodinium*. This phenomenon is mainly attributed to photoinhibition of the PSII (Warner et al. 1999). Within the PSII, numerous components are known to be susceptible to damage by the elevated temperature. These include the oxygen-evolving complex (Havaux 1993), the reaction centre (Heckathorn et al. 1998) as well as the connectivity between the light harvesting complex and the reaction centre of PSII (Schreiber and Armond 1978). Warner et al. (1999) and Lesser and Farrell (2004) have shown that the main site of photoinhibitory damage at the PSII is the D1 proteins, the loss of which is correlated with reductions in F_v/F_m . Bhagooli and Hidaka (2003) suggested that enzymes involved in the synthesis or resynthesis of the D1 protein could be affected by heat stress. Bhagooli (2013) proposed that inhibition of the Calvin-Benson cycle under elevated temperature may suppress the recovery of PSII. This enforces the suggestion that thermal stress exacerbates the pathway of cellular damage that occurs as a result of heavy metal stress, as observed in the present study.

Scleractinian corals have been reported to harbor different genetic types of *Symbiodinium*, several clades (A,

B, C, D, E, F, G, H, I) (Pochon and Gates 2010). Due to global ocean warming corals tend to change their *Symbiodinium* communities (Rowan et al. 1997; Baker 2003) with clade D as a thermally tolerant type (Rowan 2004). Members within different *Symbiodinium* clades can be further subdivided in internal transcribed spacer 2 (ITS2) types exhibiting differential thermal stress photophysiological responses (Bhagooli and Hidaka 2004b; Bhagooli 2009; Bhagooli 2010). Bielmyer et al. (2010) reported variable copper accumulation and susceptibility among three coral species harboring different *Symbiodinium* clade types, namely A3, C1 and D1a. The coral species harboring *Symbiodinium* D1a exhibited highest metal tolerance. Kuzminov et al. (2013) demonstrated differential metal toxicity in culture *Symbiodinium* of clade A1. In the present study, *F. repanda*, which has been reported to be one of the resistant coral species to bleaching events both locally (Mattan-Moorgawa et al. 2012; Bhagooli and Kaullysing 2018) and worldwide (Marshall and Baird 2000; Loya et al. 2001), was found to host *Symbiodinium* clade C. Recently, LaJeunesse et al. (2018) detailed the existing sub-cladal types (e.g. ITS2 types) and provided new names to them as distinct species. For instance, they have renamed clade C *Symbiodinium* as *Cladocopium* species. Thus, the differences in responses of *Symbiodinium* isolates to metal exposure between the present study and the other reports may be partly attributed to difference in *Symbiodinium* clade types or sub-types. Further studies on the sub-clade types, example ITS2 types, of *Symbiodinium* in *F. repanda* may provide for detailed comparison with other related studies and sub-cladal variability may imply that the present results for responses to heavy metals may not be generalized for all members of clade C. Some *Symbiodinium* types such as clade A occurring in some abundant but bleaching susceptible coral species, namely the branching *Acropora muricata*, occurring near the coast with more fluctuating temperatures, may also have some potential to acclimatize to high temperature regimes and may thus resist bleaching events (Louis et al. 2016). However, the near coast areas are also places where higher levels of both essential and non-essential metals may occur. Consequently, when the sea temperature rises gradually instead of yielding into acclimatization processes that may reduce bleaching incidences the *Symbiodinium* photophysiology may be negatively affected thus making the corals more vulnerable to thermal events in the coastal waters.

In conclusions, Cu ($\geq 50 \mu\text{g/L}$), Zn ($\geq 100 \mu\text{g/L}$) and Pb ($\geq 30 \mu\text{g/L}$) decreased the photosynthetic capacity of the *Symbiodinium* isolates from the coral *F. repanda* with more pronounced effects at higher temperature. The present study showed that a higher temperature enhanced the harmful effect of heavy metals and this lead to marked decline of the photo-physiology of symbionts of the thermally resistant coral *Fungia repanda* even under short exposure time (< 24-h). These findings suggest that coral species which may be thermally robust and are either resistant or resilient to thermal anomaly events, may be rendered photo-physiologically vulnerable to global

warming-induced mass coral bleaching/mortality events by local coastal heavy-metal contamination. It is important to note that differences in response to both essential and non-essential metals may be specific to the local *Symbiodinium* clades, and duration of metal exposure. This work provides an impetus for further investigation to determine the effects of heavy metals in the face of global warming.

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