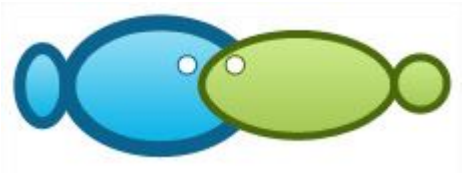

Effects of heavy metals on cell density, size, specific growth rate and chlorophyll *a* of *Tetraselmis tetrathele* under controlled laboratory conditions

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Abstract. The effects of the varying levels of mercury (Hg) and cadmium (Cd) (0, 0.1, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg L^{-1}) to the cellular density of the green microalgae *Tetraselmis tetrathele* were evaluated every 24 h for 120 h. Specific growth rate, cell sizes and chlorophyll *a* were also monitored in the 5.0 mg L^{-1} Hg and Cd and were compared to the unexposed at 0, 12, 24, 36, 48 and 120h. Results showed that the algal density of *T. tetrathele* exposed to various levels of Hg were similar with the control up to 48 h. Variations on different concentrations at different times were observed but the results suggest that *T. tetrathele* was not affected by Hg even at concentrations up to 5.0 mg L^{-1} for 48 h but started to show toxicity from 3.0 to 5.0 mg L^{-1} after 72 h and longer. Cd on the other hand also showed toxicity at 3.0, 4.0 and 5.0 mg L^{-1} beyond 24 h exposure. The specific growth rate of *T. tetrathele* exposed to both 5.0 mg L^{-1} Hg and Cd was statistically similar with those of the unexposed from 0 to 12 h and negative growth rates then followed up to 36 h. The chlorophyll *a* was significantly lower in the metal - exposed algae than did those unexposed. Chlorophyll *a* also decreased in *T. tetrathele* exposed to both heavy metals but algal cell sizes were not affected with the presence of Hg or Cd in the culture system.

Key Words: *Tetraselmis tetrathele,* mercury, cadmium, growth rate, chlorophyll *a*, cell size.

Introduction. Microalga *Tetraselmis tetrathele* under Chlorophyceae family is a very important commodity in the early larval rearing of aquatic organisms. It appears grass green due to the presence of chlorophyll *a* and *b* in its chloroplasts and the inner cell wall is made up of micro-fibril while the outer part is composed of pectin (Andersen et al 1997). It is motile due to the presence of its flagella; it is four-flagellated prasinophyte characterized by an ovoid shape with curve side views. This species is widely used in aquaculture as natural food and considered to be ideal for culture due to its high tolerance both in salinity and temperature (Das et al 2016; Farahin et al 2016; Sathasivam et al 2018). Aquaculture environment, however, and even the unpolluted waters contain toxic heavy metals like mercury (Hg) and cadmium (Cd) with a maximum of 0.1 µg L^{-1} trace amounts of Hg and dissolved or insoluble Cd primarily from discharges of industrial effluents and from atmospheric precipitation. Organic and inorganic compounds of these metals can undergo methylation through microorganisms in the sediments and the end-product can enter the food chain up to the trophic levels of aquatic organisms (Chavez et al 2006). It can be taken up by fish via the alimentary tract, gills and skin. Fish species found in the higher aquatic food chain contain the highest amount of heavy metals. The compounds formed were found to cause damage to some vital tissues and organs in fish and even to its reproduction. This includes the reduction of the viability of spermatozoa and eggs and affects the survival rate into fry at very low concentrations. Organic Hg could have a lethal concentration even at 0.025-0.125 mg L^{-1} for salmonids and 0.20-0.70 mg L^{-1} for cyprinids (Svobodova et al 1993) with allowable concentration of its organic form at 0.001 mg L^{-1} and 0.002 mg L^{-1} for

salmonids and cyprinids respectively. On the other hand, the lethal concentration of Cd is from 2 to 20 mg L^{-1} with a maximum admissible concentration of 0.0002 mg L^{-1} and 0.001 mg L^{-1} for salmonid and cyprinid cultures, respectively (Schreckenbach 1982). In humans, Hg poisoning could lead to the loss of vision, hearing, intellectual abilities and nervous disorder. Organisms in the aquatic environment including microalgae actively respond to the effects of metal poisoning. Since they could freely mobilize in their environment, contamination to other organisms are so fast, therefore it needs to be addressed immediately. Cd on the other hand is a highly toxic metal since the human body doesn't have a homeostatic control for it (Naja & Volesky 2011). It was found that ingestion of small amount of Cd would already cause nausea and headache, kidney malfunction and drop of phosphate level of the bloodstream while long term exposure could cause renal damage and bone brittleness (Jarup 2003).

Natural food is considered as the heart of aquaculture since it is essential for all aquatic organisms' survival during early stages. However, the accumulation of heavy metals in the aquatic environment could lead to their death, if not, as a carrier of these toxic substances up to the consumers. Early detection of this harmful element even at lower trophic level through algal reactions could eliminate these problems. Therefore, this study aims to investigate the effects of heavy metals Hg and Cd to the specific growth rate, size and chlorophyll *a* of green microalga *T. tetrathele* under controlled laboratory conditions.

Material and Method. This study was conducted in October 2016 at the University of the Philippines Visayas - Department of Science and Technology Aquafeeds Program Laboratory, College of Fisheries and Ocean Sciences, Institute of Aquaculture, Miagao, Iloilo Philippines. Two experiments were conducted; first on the effect of the varying levels (0.1, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0 or 5.0 mg L^{-1}) of Hg and Cd to the cellular density of the *T. tetrathele* and second, the effects of 5.0 mg L^{-1} Hg and Cd to the growth rate, cell sizes and chlorophyll *a* of the microalgae. Each experiment had a control that contained algae alone. Samplings were done every after 24 h for the first experiment and every 0, 12, 24, 36, 48 and 120 h for the second experiment. Both experiments lasted for 120 h.

Experimental species. Pure cultures of *T. tetrathele* with an initial density of $3.28x10^5 \pm 3.58x10^3$ cells mL^{-1} were obtained from Southeast Asian Fisheries Development Center Aquaculture Department at Tigbauan, Iloilo Philippines. These were viewed under the microscope to validate the purity prior to the experiment. Initial density and chlorophyll *a* were determined by manual counting under the microscope and spectrophotometrically at an absorbance of 665 and 750 nm respectively. Starters were inoculated at 30% (v/v) in 15 L polyethylene containers indoor at an axenic condition using Tongkang Marine Research Laboratory (TMRL) media by Liao & Huang (1972) but without the use of di-sodium silicate (Na_2SiO_3). Natural gas from a ring blower was supplied to the culture for aeration using rubber tubes that were individually connected to each culture container from the polyvinyl chloride pipe. The containers were incubated with a 24 h 40-watt fluorescent light for illumination.

Heavy metal-contaminated seawater preparation. Stock solutions containing mercuric chloride and cadmium chloride were prepared by dilution into triple distilled water separately. The metals were then adjusted to the desired level in each culture container by dilution to the seawater that was later used for the algal culture. To confirm the initial levels of the heavy metals, samples from the stock solutions as well as from the individual culture containers were measured using an EXACT® and LEADQUICK™ photometer ©2013 Industrial Test Systems, Inc., Rock Hill, SC, USA following the manufacturer's instruction.

Cellular density and specific growth rate (k-value) of microalgae. To determine the effect of each heavy metal to the algal density, at least 1 mL algal cell suspensions were fixed with Lugol's solution and 20 µL of the mixture were pipetted out onto the

chambers of an Improved Neubauer haemocytometer (Hasle 1978). Cells were counted using 20x objective in four corner blocks of the upper and the lower chamber of the haemocytometer. The averaged cell densities expressed in cells mL^{-1} were estimated using the formula of de la Peña & Franco (2013):

$$d\ (cells) = \frac{x}{v}$$

where x is the total count divided by the number of blocks counted and v is $1.0x10^{-4}$ mL.

The same procedures were followed to get the cellular density on the second experiment and the specific growth rate was calculated based on the data obtained. Since the growth rate of the cells is proportional to the biomass of the cells, specific growth rate had been calculated using the formula of Guillard (1975):

$$\mu = \frac{\ln N2 - \ln N1}{t2 - t1}$$

where N1 and N2 are cell numbers on times t1 and t2 respectively.

Chlorophyll a monitoring. The chlorophyll *a* of the exposed and unexposed *T. tetrathele* to the heavy metals were also measured every sampling period following the method of Boyd (1979) wherein 100 mL of the well-mixed samples were filtered to the funnel with 0.45 micron pores. These were grinded for a minute with the addition of 90% acetone to extract the pigments. For further extraction, they were refrigerated in the dark for an hour before centrifugation at 2,000-3,000 rpm and another centrifugation at 300-500 rpm after the decantation of acetone extract. Absorbance of the extract was measured at 650 nm and 750 nm against 90% acetone. Chlorophyll *a* was calculated using the formula of Vollenweider (1970):

$$Chlorophyll\ a(\mu\frac{g}{L}) = \frac{11.9\ (A665 - A750)V}{Lx1{,}000/S}$$

where A665 is the absorbance at 665 nm, A750 is the absorbance at 750 nm, L is the length of light in the spectrophotometer (cm), V is the acetone extract (mL) and S is the volume of sample filtered (mL).

Size measurement of microalgae cells. To know whether the size of the microalgae had been affected with its exposure to the heavy metals, the diameters of the 10% of the algal cells based on the samples counted were also measured every sampling period using Moticam no. 10 with Motic Images Plus 2.0 calibrated to 40x objective.

Statistical analysis. Values from algal density were expressed in base 10 logarithms prior to analysis. Data on cellular density, specific growth rate, chlorophyll *a* and cell sizes were verified for homogeneity using Levene's Test. One-way ANOVA was carried out to determine if there were significant differences between the treatments and exposure periods with $p = 0.05$. One-tailed t-test was used to compare the growth rate, chlorophyll *a* level and cell sizes of microalgae with and without the heavy metals. All statistical evaluations were carried out using the software SPSS version 16.0.

Results. The first experiment showed that the algal density of *T. tetrathele* exposed to various levels of Hg (0.1, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0 or 5.0 mg L^{-1}) were not significantly different ($p > 0.05$) from the control (algae alone) at 48 h (Table 1). However, it exhibited a significantly higher density than did those exposed to 3.0 or 5.0 mg Hg L^{-1} but remain unchanged at 72 or 96 h; at 120 h exhibited similar density to those exposed in 0.5, 1.0 or 2.0 mg Hg L^{-1}. These results suggest that *T. tetrathele* was not affected by Hg even at concentrations up to 5.0 mg L^{-1} for 48 h but started to show toxicity from 3.0 to 5.0 mg L^{-1} after 72 h and longer. Those exposed to the same level of Cd also showed to have significant differences ($p > 0.05$) from the control even at the beginning of the metal exposure (0 h) (Table 1). Lower concentrations from 0.1 to 2.0 mg L^{-1} of Cd were

then similar to the control after 24 h and all treatments showed no significant differences after 48 h. Starting from 72 h until the end of the experimental period of 120 h however, all treatments from 3.0 mg L^{-1} and beyond showed to have significantly lower density than the rest of the concentrations.

Table 1

T. tetrathele cell density (10^5 cells mL^{-1}) exposed to different levels of Hg and Cd (mg L^{-1}) for 120 h

Hg (mg L^{-1})	*Exposure time (h)*					
	0	*24*	*48*	*72*	*96*	*120*
0	2.44 ± 0.23^{a}	3.79 ± 0.66^{a}	5.29 ± 1.55	6.95 ± 0.94^{b}	6.71 ± 1.73^{b}	4.67 ± 2.31^{b}
0.1	2.63 ± 0.64^{ab}	2.09 ± 0.59^{a}	2.50 ± 0.18	4.58 ± 1.32^{b}	7.54 ± 0.47^{b}	1.18 ± 1.15^{b}
0.3	2.43 ± 0.50^{d}	1.96 ± 0.58^{ab}	2.62 ± 0.42	3.01 ± 1.41^{b}	6.25 ± 1.61^{b}	6.42 ± 0.39^{b}
0.5	1.86 ± 0.30^{bcd}	3.04 ± 0.72^{ab}	2.29 ± 0.31	4.46 ± 1.59^{b}	1.05 ± 0.71^{b}	5.88 ± 1.91^{b}
1.0	2.22 ± 0.28^{bc}	1.97 ± 0.38^{ab}	2.41 ± 1.43	4.81 ± 1.83^{b}	1.01 ± 3.08^{b}	12.48 ± 5.14^{b}
2.0	3.18 ± 0.76^{cd}	2.99 ± 0.68^{abc}	2.68 ± 0.53	1.39 ± 0.41^{b}	1.87 ± 0.24^{b}	0.50 ± 0.20^{b}
3.0	3.21 ± 0.52^{bc}	2.53 ± 0.92^{c}	2.63 ± 0.76	2.08 ± 0.36^{a}	1.61 ± 0.17^{a}	0.37 ± 0.18^{a}
4.0	2.85 ± 0.14^{bcd}	2.37 ± 0.69^{bc}	2.93 ± 1.06	2.70 ± 0.62^{a}	1.99 ± 0.26^{a}	0.65 ± 0.08^{a}
5.0	3.07 ± 0.59^{bc}	1.98 ± 0.24^{bc}	2.84 ± 0.81	2.18 ± 0.30^{a}	1.76 ± 0.18^{a}	0.57 ± 0.04^{a}
Cd (mg L^{-1})						
0	1.49 ± 0.27^{a}	1.98 ± 0.19^{a}	3.16 ± 0.28	5.27 ± 1.27^{b}	4.47 ± 1.78^{b}	3.22 ± 0.90^{b}
0.1	1.87 ± 0.25^{ab}	1.99 ± 0.56^{a}	4.02 ± 1.02	3.93 ± 0.22^{b}	2.69 ± 1.39^{b}	0.57 ± 0.50^{a}
0.3	3.23 ± 0.24^{d}	2.30 ± 0.75^{ab}	4.39 ± 3.39	5.78 ± 0.72^{b}	2.22 ± 0.65^{b}	1.02 ± 0.83^{a}
0.5	2.43 ± 0.40^{bcd}	2.22 ± 0.84^{ab}	3.54 ± 2.83	6.34 ± 1.00^{b}	4.91 ± 1.46^{b}	3.58 ± 1.78^{b}
1.0	2.40 ± 1.00^{bc}	2.14 ± 0.49^{ab}	3.88 ± 1.32	4.65 ± 1.15^{b}	4.68 ± 1.23^{b}	2.55 ± 0.28^{b}
2.0	2.83 ± 0.30^{cd}	3.08 ± 0.28^{abc}	3.34 ± 0.61	3.63 ± 0.74^{b}	3.19 ± 1.56^{b}	3.24 ± 1.46^{b}
3.0	2.19 ± 0.12^{bc}	4.29 ± 1.08^{c}	2.18 ± 0.83	1.93 ± 1.01^{a}	0.53 ± 0.27^{a}	0.63 ± 0.63^{a}
4.0	2.37 ± 0.27^{bcd}	3.35 ± 0.41^{bc}	2.48 ± 0.47	1.27 ± 0.81^{a}	0.57 ± 0.36^{a}	0.33 ± 0.11^{a}
5.0	2.30 ± 0.07^{bc}	3.27 ± 0.42^{bc}	2.80 ± 0.55	1.43 ± 0.43^{a}	1.10 ± 1.02^{a}	0.35 ± 0.15^{a}

*Values with the same superscripts in the same column are not significantly different. All values were expressed as Mean±SD ($p = 0.05$), $n = 3$.

Algal growth rate (k-value). Results showed that the specific growth rate of *T. tetrathele* when exposed to 5.0 mg L^{-1} Hg was similar with those of the unexposed microalgae from 0 to 12 h (Table 2). Negative growth rates then followed up to 36 h. Significant differences ($p < 0.05$), wherein the algae with Hg were lower, between the two treatments were observed at 48 h while negative growth rate occurred again at the end of the exposure period. On the other hand, when two cultures, with and without Cd were compared, it showed that significant differences occurred during 24 and 36 h exposure ($p < 0.05$). In addition, the treatment with Cd also had significantly lower growth rate compared to the treatment without Cd during 48 and 120 h (Table 2). Each treatment was also analyzed separately to determine the effects of the exposure time to the algal growth rate. It showed that in algae with Cd, only during 12 h had significantly higher ($p < 0.05$) growth rate while the rest of the exposure times (24, 36, 48 and 120 h) were similar.

Table 2

Specific growth rate (10^6 cells h^{-1}) of *T. tetrathele* with and without 5.0 mg L^{-1} Hg or Cd exposed for 120 h

Treatment	*Exposure time (h)*				
	12	*24*	*36*	*48*	*120*
T. tetrathele + Hg	8.18 ± 1.74	-3.37 ± 1.49	-5.73 ± 2.00	$1.84\pm3.75^{*}$	-0.71 ± 0.11
T. tetrathele + Cd	6.89 ± 1.23	$-0.82\pm1.48^{*}$	$-1.11\pm0.63^{*}$	$1.36\pm2.19^{*}$	$-1.21\pm0.48^{*}$
T. tetrathele	7.02 ± 0.43	$-2.25\pm0.98^{*}$	$-0.11\pm1.10^{*}$	3.28 ± 0.95	-1.99 ± 0.09

*Asterisks indicate statistically significant differences compared with *T. tetrathele* alone (* with significant difference, t-test). Values expressed as Mean±SD ($p < 0.05$), $n = 3$.

Chlorophyll a of microalgae. The microalgae exposed to Hg exhibited significantly lower chlorophyll *a* than did those unexposed (Figure 1A). The differences in chlorophyll *a* occurred from the start of the experiment until 120 h exposure. The microalgae that are not exposed to Hg exhibited higher amount of chlorophyll *a* throughout the culture period except at the beginning of the experiment. In contrast, those exposed to Hg increased at 12 h, but decreased at 24 h onwards. *T. tetrathele* exposed to 5.0 mg L^{-1} Cd on the other hand showed similar amount of chlorophyll *a* with those that were not exposed from the beginning of the experiment up to 48 h (Figure 1B). During 120 h exposure however, those microalgae with Cd contained higher amount of chlorophyll *a* (109±38.7 µg L^{-1}) compared to those without Cd (13.1±9.92 µg L^{-1}). Both treatments had similar amount of chlorophyll *a* during the periods 12, 24, 36 and 48 h and both also significantly decreased after 120 h ($p < 0.05$).

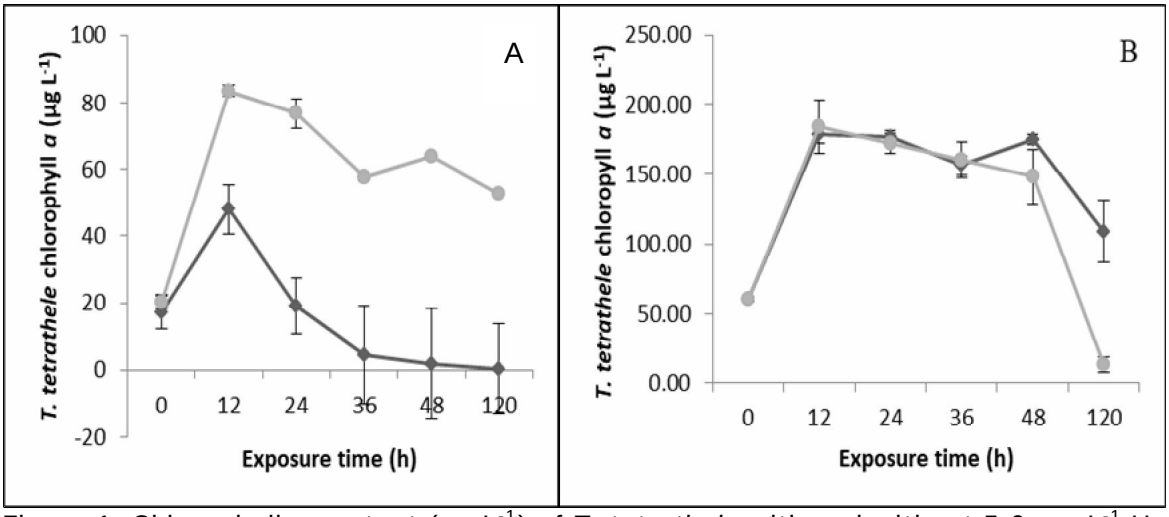

Figure 1. Chlorophyll *a* content (µg L^{-1}) of *T. tetrathele* with and without 5.0 mg L^{-1} Hg (A) and Cd (B) with respect to time (h). Dark lines represent heavy metal + algae while light lines represent algae alone. Data presented as Mean±SD ($p < 0.05$), n = 3.

Algal cell sizes exposed to heavy metals. The lengths of the algal cells were measured to know if it is affected by the two heavy metals. Results showed that the algal cell sizes were similar regardless of whether it is exposed to Hg or Cd and regardless of the exposure period (Figures 2 and 3). *T. tetrathele* diameters exposed to Hg and Cd were 12.7±0.63 µm and 12.9±0.10 µm respectively.

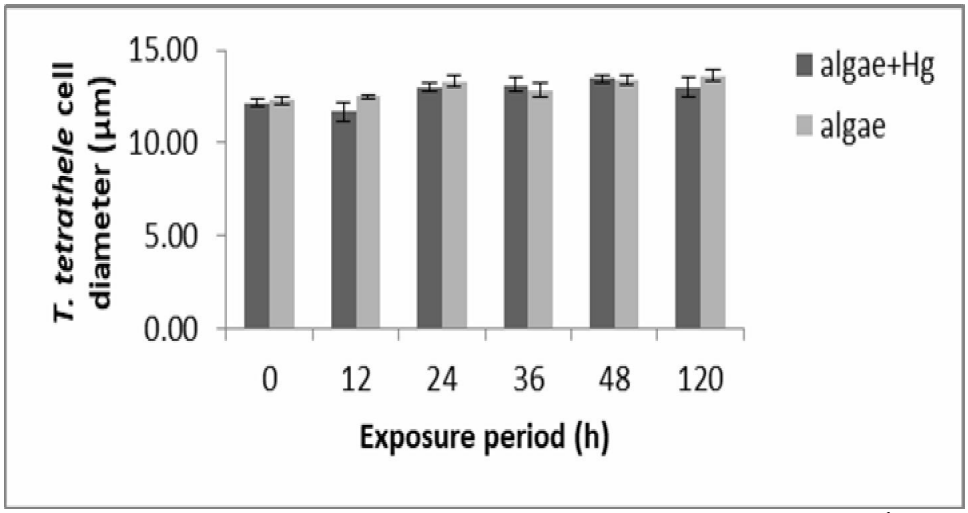

Figure 2. *T. tetrathele* cell diameters (µm) exposed to 5.0 mg L^{-1} Hg. Data presented as Mean±SD ($p < 0.05$), n = 3.

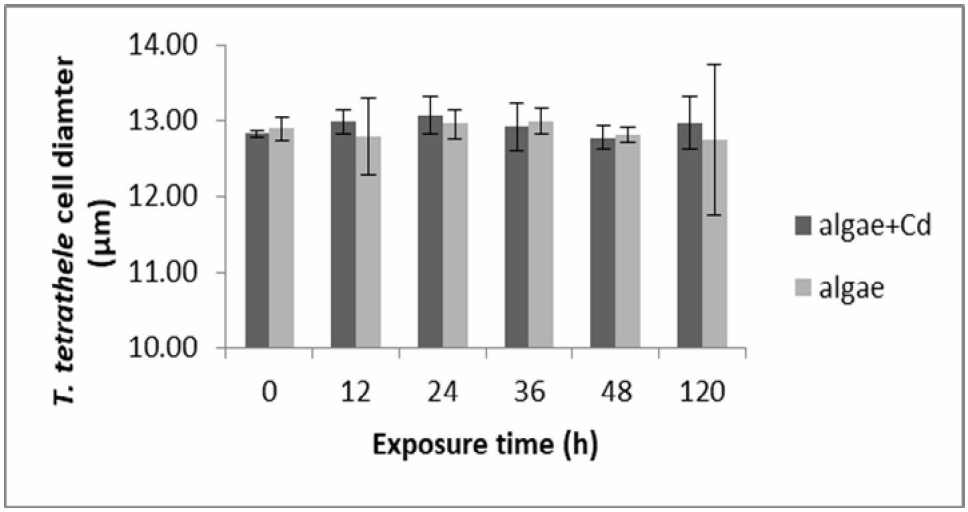

Figure 3. *T. tetrathele* cell diameters (µm) exposed to 5.0 mg L^{-1} Cd. Data presented as Mean±SD ($p < 0.05$), n = 3.

Discussion. Microalgae are recorded to be very sensitive to their environment and inorganic pollutants like heavy metals change their overall metabolism. The toxic effects of these metals may be caused by the blocked functional groups of important molecules like enzymes and transport systems for essential nutrients and ions, the displacement and/or substitution of essential metal ions from biomolecules and functional cellular units and the induction of cellular generation of reactive oxygen species (ROS) such as peroxide anion, hydrogen peroxide, singlet oxygen and hydrogen radical (Kaplan 2013). Proteins, lipids and nucleic acids could be oxidized by high levels of ROS resulting in the modification and inactivation of enzymes as well as disruption of cellular and organellar membrane integrity leading to algal death (Kaplan 2013; Le Faucheur et al 2013).

In the present study, *T. tetrathele* was not affected by Hg even up to 5.0 mg L^{-1} at 48 h based on its cellular density. It started to be toxic from 3.0 to 5.0 mg L^{-1} at 72 h and beyond. Furthermore, the algal specific growth rate also decreased between 12-24 h and 48-120 h. Hg concentrations of 0.10 to 0.20 mg L^{-1} can reduce the cellular density of *T. chuii* especially at 48 h (Cordero et al 2005). It could be toxic to other microalgal species even at a concentration of .005 mg L^{-1} Hg (Shanab et al 2012). On the other hand, this study revealed that *T. tetrathele* had higher sensitivity to Cd. Reactions have occurred as soon as the metal was added to the culture. At 24 h exposure however, no significant differences were observed between the cellular densities of *T. tetrathele* with Cd and without Cd. The algae probably "avoided" the toxic metals during this period from entering into its cells thus it was able to multiply even with 5.0 mg L^{-1} Cd (Levy et al 2008). Starting from 72 h-exposure to 3.0 mg L^{-1} Cd and above, toxicity to the algae was already observed. This is because Cd concentrations greater than 1 mg L^{-1} is already toxic to the algae (Satoh et al 2005).

The organelles of the microalgae including the mitochondria and the chloroplast are the sites for photosynthetic activities inside the cells (Le Faucheur et al 2013). Metals bind on these same sites, therefore, it is expected that chlorophyll *a* production of microalgae will also decrease upon exposure to heavy metals. The chlorophyll *a* of *T. tetrathele* exposed to 5.0 mg L^{-1} Hg in this study appeared to be significantly lower than the unexposed throughout the culture period. Hg concentration of 5-10 µg mL^{-1} could inhibit the chlorophyll *a* synthesis in microalgae (Matson et al 1972; Shanab et al 2012; Gomez-Jacinto et al 2015). It strongly inhibits photosynthetic electron transport reactions in algae and higher plants (Krupa & Baszynski 1995). It has toxic effects on the photosynthetic electron chain that could decrease the capacity of the photosystem to dissipate the excitation light energy via the photochemical pathway (Samson & Popovic 1990; Nahar & Tajmir-Riahi 1995; Graevskaia et al 2003). Results of the present study was in contrast with those of Rosko & Rachlin (1977) on the freshwater microalgae *Chlorella vulgaris* that showed an increase in chlorophyll *a* content after 33 days exposure to 2.40 mg L^{-1} Hg. They concluded that this metal exerted a greater effect on

cell division than on chlorophyll *a* content. In the present study, both the cellular density and the chlorophyll *a* were affected. In contrast, the chlorophyll *a* of *T. tetrathele* exposed to Cd in this study was similar to those that were not exposed up to 48 h. Even though both treatments decreased at 120h, those with Cd contained higher amount of chlorophyll *a* than those without Cd. This showed that the algal strain used in this study requires more exposure time to adapt to the presence of Cd than those used by Satoh et al (2005) which had a continuously increasing chlorophyll *a* even with 6.25 mg L^{-1} Cd for 72 h. Nevertheless, *T. tetrathele* in this study probably had an internal detoxification mechanism involving the synthesis of metal-binding peptides for detoxification of Cd (Perez-Rama et al 2002).

The specific surface area of the microalgae has a direct effect to metal removed in the water in relation to the membrane transporters, metal mass balance, and the rate of the carriers, time and the algae's specific growth rate (Ting et al 1991). In this study, the algal cell sizes were statistically similar regardless of whether it is exposed or not to the heavy metal Hg. This result was in agreement with those of Shanab et al (2012) who have worked on *Pseudochlorococcum typicum* and found unchanged morphological features including cell sizes after heavy metal treatment.

Conclusions. The algal densities of *T. tetrathele* exposed to various levels of Hg were similar with the control up to 48 h. At different concentrations and times, *T. tetrathele* was not affected by Hg exposure even up to 5.0 mg L^{-1} for 48 h but started to show toxicity from 3.0 to 5.0 mg L^{-1} after 72 h and longer. Cd also showed toxicity at 3.0, 4.0 and 5.0 mg L^{-1} beyond 24 h exposure. The specific growth rate of *T. tetrathele* exposed to both Hg and Cd at 5.0 mg L^{-1} were similar with those of the unexposed from 0 to 12 h and negative growth rates then followed up to 36 h. The chlorophyll *a* decreased in the metal-exposed algae than did those unexposed but algal cell sizes in the culture system were not affected with the presence of these heavy metals.

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