

INFLUENCE OF LEAD ON GROWTH AND PHYSIOLOGICAL CHARACTERISTICS OF A FRESHWATER GREEN ALGA *CHLORELLA* SP.

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Received: 13th December 2017

Revised: 18th October 2018

Accepted: 28th October 2018

DOI: <https://doi.org/10.22452/mjs.vol37no2.1>

ABSTRACT Pollution caused by heavy metals poses a serious challenge to ecosystems. Microalgae are microscopic algae that form the base of the food chain in aquatic ecosystems. Heavy metals present in soils can leach into aquatic ecosystems, leading to bioaccumulation by organisms through the food chain, and exerting toxic effects on microalgae, the primary producers. This study reports the response of the alga *Chlorella* sp. to lead stress over experimental exposures of 11 days. *Chlorella* sp. were grown and exposed to different lead concentrations (0, 10, 25, 50, 75 and 100 mg/L) at 18°C. The experiments were conducted in triplicate. Growth rate of microalgae was determined by measuring the optical density at 620 nm (OD₆₂₀) and chl-*a* concentration. The data obtained showed that lead affects the physiological characteristics (growth, chl-*a* and carotenoids) and protein content of *Chlorella* sp. Exposure to increasing lead concentrations resulted in decreasing specific growth rate (μ), chlorophyll-*a*, carotenoid and protein contents. However, the data obtained indicate that *Chlorella* sp. can tolerate the toxicity of lead even at high concentration.

Keywords: *Chlorella*, heavy metals, lead, specific growth rate, protein

ABSTRAK Pencemaran yang disebabkan oleh logam berat menimbulkan cabaran serius terhadap ekosistem. Mikroalga adalah alga mikroskopik yang membentuk asas rantaian makanan dalam ekosistem akuatik. Logam berat yang terdapat di dalam tanah boleh meluap ke dalam ekosistem akuatik, yang membawa kepada bioakumulasi oleh organisma melalui rantai makanan, dan menghasilkan kesan toksik pada mikroalga, pengeluar utama rantaian makanan. Kajian ini melaporkan tindak balas alga *Chlorella* sp. terhadap tekanan plumbum pada pendedahan eksperimen selama 11 hari. *Chlorella* sp. telah dikultur dan didedahkan kepada kepekatan plumbum yang berbeza (0, 10, 25, 50, 75 and 100 mg/L) pada suhu 18°C. Eksperimen ini dijalankan dalam triplikasi. Kadar pertumbuhan mikroalga ditentukan dengan mengukur kepadatan optik pada 620 nm (OD₆₂₀) dan kepekatan klorofil-*a*. Data yang diperolehi menunjukkan bahawa plumbum mempengaruhi ciri-ciri fisiologi (pertumbuhan, klorofil-*a* dan karotenoid) dan kandungan protein *Chlorella* sp. Pendedahan kepada peningkatan kepekatan plumbum mengakibatkan penurunan kadar pertumbuhan spesifik (μ), klorofil-*a*, karotenoid dan kandungan protein. Walau bagaimanapun, data yang diperolehi menunjukkan bahawa *Chlorella* sp. mampu bertoleransi terhadap ketoksikan plumbum walaupun pada kepekatan tinggi.

Kata kunci: *Chlorella*, logam berat, plumbum, kadar pertumbuhan spesifik, protin

INTRODUCTION

Heavy metal pollution is a serious environmental issue that is harming natural ecosystems and human health. Heavy metals can enter the environment through air, soil or water. Increasing amounts of heavy metals have been released into estuarine ecosystems over the last century (Jung, 2017; Penuelas & Filella, 2002; Nriagu & Pacyna, 1988). Lead, cadmium and mercury are examples of non-essential heavy metals that can cause harm to living organisms even at low concentration. Heavy metals such as zinc and copper are essential micronutrients for living organisms. However, toxicity occurs when the concentration of a particular metal ion in the environment exceeds a specific threshold level. Industrial wastes and urban wastes that contain heavy metals are often released into rivers and coastal regions leading to aquatic pollution (Jung, 2017; Wang et al., 2007).

Heavy metal-containing compounds cannot be degraded by microorganisms, unlike many organic compounds. When these heavy metals are converted into stable and permanent pollutants, build-up in soils and sediments will occur (McComb et al., 2014). These heavy metals can cause adverse effects to organisms living in aquatic ecosystems as they enter the food chain and bioaccumulation takes place through successive trophic levels. When heavy metals accumulate in marine biota such as fish and other marine animals, they can then be consumed by humans or domestic animals (through feed), with harmful consequences to human or animal health (McComb et al., 2014; Shanab et al., 2012; Kong et al., 1995).

Lead is a toxic heavy metal that occurs naturally in the Earth's crust. The natural levels of lead in soil range between 0.5 and 10 ppm (USEPA, 2003). This metal

is extensively used in industrial applications such as batteries and paints (Yun et al., 2011). Lead may enter the environment from these past and current uses and resulted in substantial increases in lead levels in the environment. Lead from anthropogenic sources may result in concentrations exceeding 10,000 ppm (USEPA, 2003). Individuals working near lead mining, smelting and construction locations are particularly exposed to lead as it can enter the human body through ingestion and inhalation. When the blood lead levels exceeds 25 $\mu\text{g/dL}$, this causes adverse effects such as neurological damage and chronic nephropathy (Mason et al., 2014; Papanikolaou et al., 2005). Lead can cause severe harm to aquatic life even at low concentration of 10 ppm (Kaplan, 2013). It exerts toxic effects by forming ligands with other metabolites, competing with other metabolites by binding to functional groups, or by replacing other metals with the same ionic charges causing changes in enzymatic activities and disruption of cellular processes (Báscik-Remisiewicz et al., 2009; Awad et al., 2005).

Microalgae are diverse organisms that have rapid growth rates and productivity and are able to live in harsh conditions due to their unicellular or simple multicellular structure (Li et al., 2008a, b). Microalgae are the primary producers at the base of the aquatic food chain and are the first organisms affected by heavy metal pollution. Microalgae are commonly used as biosensors in environmental analyses and monitoring due to their high sensitivity and specificity in detecting heavy metal pollutants (Mata et al., 2009; Védrine et al., 2003). Studies have shown that microalgae possess extracellular and intracellular mechanisms to resist metal toxicity (Scheidegger et al., 2011; Perales-Vela et al., 2006), including the ability to absorb a certain amount of heavy metals. Heavy metals are able to enter algal cells via active transport or endocytosis, with help from

chelating proteins such as phytochelatin and class II metallothioneins thus, reducing heavy metal toxicity. These chelating proteins form complexes with heavy metals and transfer them to intracellular vacuoles (Perales-Vela et al., 2006). However, toxicity occurs when the amount of heavy metals taken up exceeds a critical concentration. The binding of heavy metal ions to sulphhydryl groups in the protein cysteine, disruption of protein structure or displacement of essential elements will, eventually, affect the physiological and biochemical processes of the microalgae (Arunakumara & Zhang, 2008).

Chlorella contains important biochemical components such as chlorophyll-*a* (chl-*a*), carotenoids, proteins, lipids and carbohydrates. These green algae are consumed by humans as health supplements in the form of tablets, liquid extracts or powder. Species of *Chlorella* are also commonly used to assess the responses of aquatic ecosystems to heavy metal pollution by providing essential information on the toxic effects of specific heavy metals on metabolic processes (Sears, 2013). In this study, *Chlorella* was chosen as a model to conduct toxicity testing due to its rapid growth rate and productivity, high sensitivity to various heavy metals, and high ability to adapt to different environmental conditions (Carfagna et al., 2011; Li et al., 2008a, b; Nyholm & Kallqvist, 1989).

The aim of this study was to measure growth, chl-*a* concentration, carotenoid and protein content of *Chlorella* sp. exposed to different lead concentrations (0, 10, 25, 50, 75 and 100 mg/L) over an 11 days experimental period. The percentage of inhibition of lead in *Chlorella* sp. was also determined.

MATERIALS AND METHODS

Algal Culture

A culture of *Chlorella* sp. (152069) was purchased from Carolina Biological Supply Company (USA). This was sub-cultured in Bold's Basal Medium (BBM) and maintained in a temperature-controlled growth chamber (Protech Growth Chamber, Tech-Lab Scientific Sdn. Bhd., Malaysia) set at 18°C. Illumination was provided by cool fluorescent lamps (42 $\mu\text{mol}/\text{m}^2/\text{s}$) with 12-12 light-dark cycles.

Experimental Design

A stock solution of 1000 mg/L Pb(II) was prepared by dissolving $\text{Pb}(\text{NO}_2)_3$ in distilled water and filter sterilization. The working solutions were diluted to the desired concentrations during the experiments. At a constant temperature of 18°C, triplicate flasks containing algal cultures were exposed to different lead concentrations (0, 10, 25, 50, 75, and 100 mg/L) for 11 days (stationary phase) to study their growth responses, chl-*a*, carotenoid, and protein content.

Growth Measurements

The inoculum (10 %) used was from exponential phase cultures standardized at an optical density at 620 nm (OD_{620}) of 0.2. The algal cultures were grown in 500 mL flasks and were exposed to different lead concentrations as described above. The cultures were sampled on day 0, 2, 4, 7, 9 and 11 to determine growth rate. Two parameters were used to monitor growth of cultures, OD_{620} and chl-*a* concentration. The latter was measured using a spectrophotometric method after extraction of the filtered cells (glass-fibre filters, 0.45 μm) in acetone (Parsons & Strickland,

1963). Specific growth rate (μ , d^{-1}) based on OD_{620} was calculated using the following formula:

$$\mu (d^{-1}) = (\ln N_2 - \ln N_1) / (t_2 - t_1)$$

Where N_2 is OD_{620} at t_2 , N_1 is OD_{620} at t_1 , and t_2 and t_1 are times within the exponential phase. The cells were harvested at the end of the experiment by filtration for determination of the protein content.

Protein Analysis

Proteins were extracted in 0.5 N NaOH (80°C, 30 min) and the concentration determined by the dye-binding method (Bradford, 1976).

Percentage of Inhibition

The following equation was used to calculate the percentage of inhibition based on specific growth rate (μ) of the control and treatments.

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100\%$$

Where $I_{\mu i}$ = Percentage of inhibition for test concentration i , μ_i = Mean specific growth rate for test concentration i and μ_c = Mean specific growth rate for test concentration i .

Statistical Analyses

IBM SPSS Statistics 20.0 was used to conduct statistical analysis. Analysis of Variance (ANOVA) was used to test for overall differences between treatments, followed if significant by pairwise Tukey comparisons. The different concentration of lead was used as the independent variable while specific growth rate, final chl-*a* concentration, carotenoid, protein and percentage of inhibition were used as dependent variables. Differences were considered significant at $p < 0.05$.

RESULTS

Growth Trends

Based on the specific growth rate (μ), a decreasing trend in μ was observed as the concentration of lead increased from 0 mg/L to 100 mg/L (Figure 1). The cultures grown under control condition (0 mg/L lead) exhibited the highest μ ($0.286 \pm 0.003 d^{-1}$) while those exposed to 100 mg/L showed the lowest μ ($0.257 \pm 0.003 d^{-1}$). The μ of control and lead concentration of 10 mg/L did not differ significantly. Although there was a limited but significant decrease in growth with increasing lead concentrations, the algae could clearly tolerate and grow at all the lead concentrations tested over the 11 days duration of the experiment.

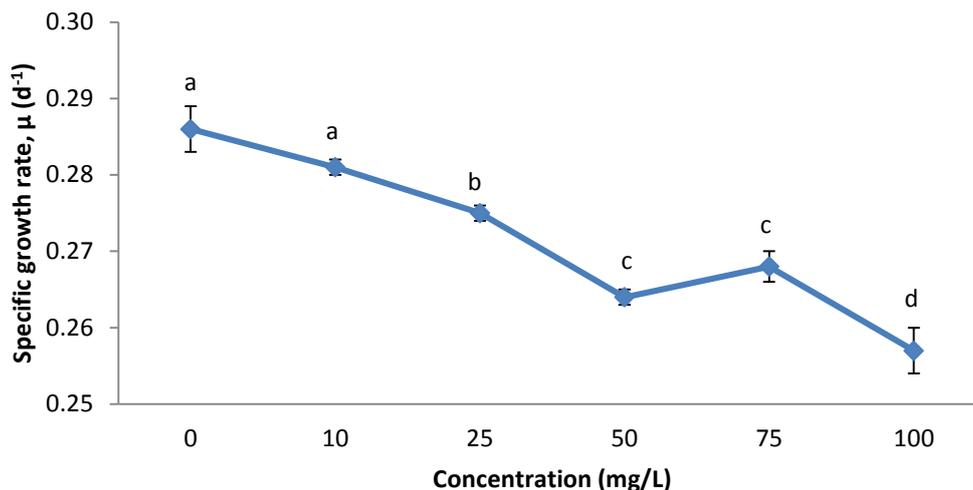


Figure 1: Specific growth rate (μ , d^{-1}) based on OD_{620} of *Chlorella* sp. grown at different lead concentrations. Data represent mean values of triplicate cultures and error bars are standard deviations. Different letters indicate significant differences at $p < 0.05$.

Final Chl-a and Carotenoid Contents

Lead concentrations of 0 mg/L (control) led to the highest final chl-a concentration (16.07 ± 0.74 mg/g DW) while the highest lead concentration of 100 mg/L caused a marked decreased (64.28%) in final chl-a concentration (5.74 ± 0.25 mg/g DW)

(Figure 2). A decreasing trend in the final chl-a concentration was clear as the lead concentration increased. A similar but less strong pattern was apparent in carotenoid content, with the highest content in the control (0.72 ± 0.08 μ g/mL) and the lowest at a lead concentration of 100 mg/L (0.57 ± 0.03 μ g/mL).

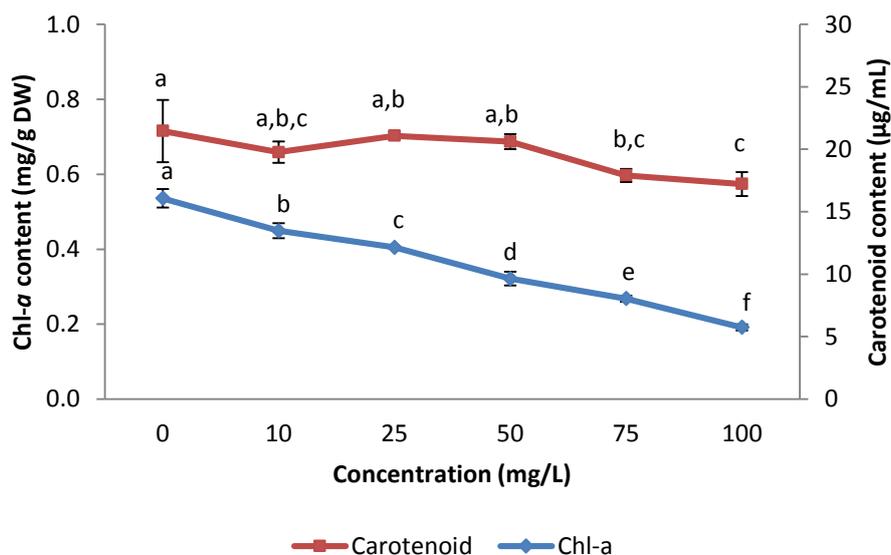


Figure 2: Final chl-a and carotenoid contents of *Chlorella* sp. grown at different lead concentrations. Data represent mean values of triplicate cultures and error bars are standard deviations. Different letters indicate significant differences at $p < 0.05$.

Protein Content

Cultures grown at zero or low lead concentrations (0, 10, 25 mg/L) showed

similar protein content, significantly greater (c. 40 %) than those grown at lead concentrations of 50, 75 and 100 mg/L (Figure 3).

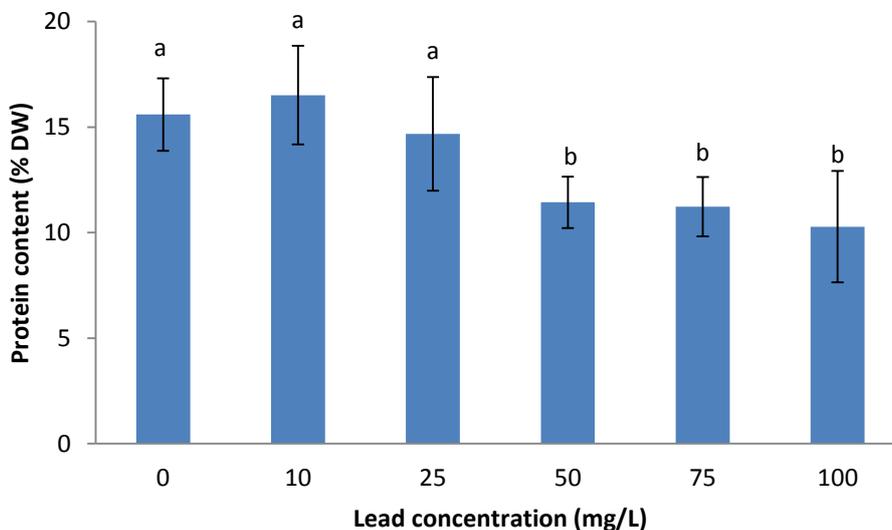


Figure 3: Protein content (% DW) of *Chlorella* sp. grown at different lead concentrations. Data represent mean values of triplicate cultures and error bars are standard deviations. Different letters indicate significant differences at $p < 0.05$.

Percentage of Inhibition of Growth

Percentage of inhibition of growth rate of *Chlorella* sp. grown at different lead

concentrations is shown in Figure 4. All lead concentrations to some extent inhibited growth, varying between only slight inhibitions (1.75 %) at 10 mg/L to 10.14 % at 100 mg/L.

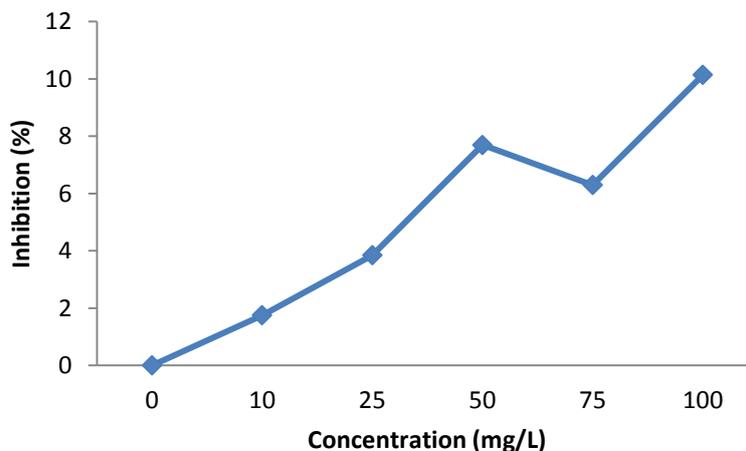


Figure 4: Percentage of inhibition of growth of *Chlorella* sp. grown at different lead concentrations.

DISCUSSION

Specific growth rate (μ) is used to determine the ability of microalgae growth in lead-influenced environments. Here, cultures grown under the highest lead concentration (100 mg/L) had the lowest μ , consistent with other studies that have reported that the μ of *Chlorella sorokiniana* (Carfagna et al., 2013), *C. vulgaris* and *C. protothecoides* (Zhang et al., 2013) declined with exposure to increasing lead concentration.

Our data indicate that the *Chlorella* sp. strain examined is able to tolerate the toxicity of lead even at higher concentration (100 mg/L). Other algae such as *Phormidium ambiguum*, *Pseudochlorococcum typicum* and *Scenedesmus quadricauda* var. *quadrispina* are also able to tolerate lead concentrations up to 100 mg/L (Shanab et al., 2012). This may be due to a variety of mechanisms possessed by microalgae to maintain metal homeostasis and prevent toxicity. At intracellular level, heavy metals may enter cells through micronutrient transporters, with accumulation inside the cells being via active processes. Once within the cell, they can bind to peptides or polyphosphate bodies to form organometallic complexes. The synthesis of peptides including phytochelatins and class II metallothioneins can be enhanced by metal ions such as Cd^{2+} and Pb^{2+} (Kaplan, 2013; Perales-Vela et al., 2006). Sulphide ions (S^{2-}) that can also be present in the organometallic complex will also help in stabilizing the complex, enhancing the detoxification effect. The organometallic complex may then be transported to specific cellular compartments such as vacuoles to help in controlling the cytoplasmic concentration of heavy metal ions and, thereby, protect against their toxicity (Kaplan, 2013; Perales-Vela et al., 2006).

A high concentration of heavy metal ions can exert a great impact on physiological functions of algae such as growth and cell division (Wang & Chen, 2009). The reduction in growth rate of *Chlorella* sp. at highest lead concentration examined (100 mg/L; Figure 1) might be due to interaction of the metal ion with the fat layer on the cell surface of microalgae at extracellular level (Chu & Hashim, 2007). On the surface of microalgae cells, there are a number of functional groups exposed such as $-\text{NH}_2$, $-\text{COO}^-$ and $-\text{SH}$, that function as metal binding sites (Kumar et al., 2014). Heavy metal ions binding to these sites can then pass through the cell membrane to enter the cytoplasm (Kumar et al., 2014). This can cause disruption to the microalgal intracellular structure as well as disrupting physiological and biochemical processes (Kumar et al., 2014; Al-Hejuje, 2008).

When algal cells are exposed to lead, this results in reduced energy supply as well as negative impacts on intracellular enzyme systems such as lipase and cellulase (Sander & Murthy, 2009; Awasthi & Das, 2005). These effects can depress the rate of cell division or cause damage to the cell membrane. Cell membrane damage can lead to uncontrollable influx or efflux of electrolytes or vital ions leading to either an excess or a deficiency in the cytoplasm, affecting growth (Awasthi & Das, 2005).

Chlorophyll is the most important pigment in microalgae cells, key to absorbing the solar energy required for photosynthesis. In this study, exposure to 100 mg/L lead concentration resulted in an approximately 64% reduction in chl-*a* concentration (Figure 2). Other studies have shown that increasing lead concentration result in decreased rates of photosynthesis associated with declining of chl-*a* concentrations in *Chlorella* species (Zhang et al., 2013; Shanab et al., 2012). When microalgae are exposed to lead, the

metal ion's oxidative potential causes inhibition of key enzymes that play a crucial role in the CO₂-fixation cycle. Any decrease in the activity of these essential enzymes will reduce ATP synthesis in CO₂-fixation, where ATP and NADPH are primary requirements for CO₂-fixation. Reduction in ATP synthesis will reduce the production of ATP and NADPH and thus, impact the photosynthetic rate (Muwafq & Bernd, 2006; Awasthi & Das, 2005).

Exposure to heavy metals influences the electron transfer systems in mitochondria and chloroplasts. This will hinder the electron flow on both the acceptor and donor side of PSII, affecting the biochemical processes involved in photosynthesis (Al-Hejuje, 2008). Moreover, it can be assumed that exposure to heavy metals may lead to substitution or impairing of the uptake of important elements for photosynthetic pigments such as magnesium and potassium. Disruption of cation exchange will cause alteration of chloroplast ultrastructure and lead to damage of thylakoid membranes and reaction centres (Dao & Beardall, 2016; Carfagna et al., 2013), affecting the light harvesting pigments. Transformation of shape and organization of thylakoids indicates damage to the photosynthetic apparatus, leading to inhibition of PSII and a decrease in the chlorophyll content and chlorophyll *a/b* ratio (Neelam & Rai, 2003). A recent study by Dao & Beardall (2016) also reported that lead affected both the reaction centres and the antennae of *Chlorella* and *Scenedesmus*, and therefore impacted negatively on their PSII activity and caused alterations in PSII heterogeneity.

Carotenoids are light-harvesting pigments that possess antioxidant properties that suppress triplet state chlorophyll molecules, quench toxic free radicals and help to restore physiological balance. The total carotenoid content of *Chlorella* sp. under the treatments imposed

in the current study showed a similar pattern to the final chl-*a* concentration (Figure 2). Cultures grown at the highest lead concentration (100 mg/L) generated a slightly lower carotenoid level (0.57 ± 0.03 µg/mL) compared to other concentrations. Similar findings have also been reported in another species (*Oscillatoria boryana*), which showed decreasing carotenoid content when exposed to increasing concentration of heavy metals (Priyadarshani & Rath, 2012). However, the absolute impact of lead on carotenoid content was not large. Other studies have reported that Chlorophyceae such as *Chlorella* and *Scenedesmus* are able to accumulate carotenoids such as canthaxanthin and astaxanthin under stressful conditions (Guedes et al., 2011; Takaichi, 2011).

Exposure to heavy metals can result in intermittent intracellular oxidative stress conditions resulting from the accumulation of reactive oxygen species (ROS) (Mittler, 2002; Pinto et al., 2003). When microalgae are exposed to stressful conditions, they synthesize additional secondary carotenoids via carotenogenesis (Faraloni & Torzillo, 2017; Lemoine & Schoefs, 2010). Oxidative stress induced by heavy metal exposure has been demonstrated to affect astaxanthin synthesis (Takaichi, 2011). Astaxanthin could play a crucial role in responding to stress in microalgae, as accumulation of astaxanthin is assumed to be a defensive reaction to stress, protecting the microalgae against oxidative damage (Gao et al., 2013; Guedes et al., 2011).

Cultures grown at zero or low lead concentrations produced the highest percentage of protein content (Figure 3). High lead concentrations slightly but significantly decreased the protein content of the microalgae, as also reported by Carfagna et al. (2013). Proteins within microalgal cells are used as an emergency source of nitrogen and sulphur to promote cell growth (Carfagna et al., 2013). It is also

assumed that any limitation of sulphur availability will reduce protein biosynthesis. Ultimately this may cause failure of microalgae to perform photosynthesis in the reaction centre protein of PSII in the thylakoid membrane, affecting the growth and chl-*a* concentration of microalgae (Pandey et al., 2013). In addition, the inability of *Chlorella* sp. to accumulate proteins after exposure to high lead concentration might be caused by acute oxidative stress induced by heavy metal excess in cells (Bajguz, 2011; Arunakumara & Zhang, 2008).

The data obtained in the current study demonstrate that the strain of *Chlorella* sp. studied was capable of tolerating high lead concentrations of at least 100 mg/L with only limited reduction in performance over an 11 days exposure period. We suggest that various mechanisms by which microalgae are known to mitigate the effects of heavy metal stress, such as binding of metal ions to peptides and sites located on the surface of microalgal cells, were important in this resistance. Given the potential of *Chlorella* sp. in bioremediation, more detailed studies should be conducted including different parameters such as pH and temperature to understand the detailed mechanisms of heavy metal regulation in these microalgae. Furthermore, computational approaches such as docking simulation or dynamics simulation can be added to further substantiate the response of microalgae at the molecular level (Brautigam et al., 2011).

ACKNOWLEDGEMENTS

This project is supported by the School of Biosciences, Taylor's University under its Final Year Project Programme. We thank Prof. Dr. Peter Convey for comments which helped to strengthen the manuscript.

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