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Comparison of Sensitivity of Tropical Freshwater Microalgae to Environmentally Relevant Concentrations of Cadmium and Hexavalent Chromium in Three Types of Growth Media

Thilini Munagamage[1] · I. V. N. Rathnayake[1] · A. Pathiratne[2] · Mallavarapu Megharaj[3]

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Abstract

Sensitivity of tropical freshwater microalgae (*Mesotaenium* sp., *Chlorococcum* sp. and *Scenedesmus* sp.) to environmentally relevant concentrations of hexavalent chromium (Cr^{6+}) and cadmium (Cd^{2+}) was compared individually in three growth media viz. Bold's Basal Medium (BBM), Test Medium 1 (TM1) and Test Medium 2 (TM2) based on fluorescence reduction. Free metal content of growth media was determined by Visual MINTEQ (version 3.1). After 24 h, relative fluorescence of microalgae in the three media decreased with increased metal concentration showing a concentration dependent graded toxicity response. All microalgae were more sensitive to the metals when grown in TM1, when compared, more sensitive to Cr^{6+} than Cd^{2+}. Metal speciation indicated that TM1 and TM2 media have higher percentage of bioavailable Cd^{2+} than BBM, and chromium was present mainly as CrO_4^{2-} and $HCrO_4^-$. The results suggest that the TM1 medium is more suitable under short term exposure of microalgae to metals in environmental monitoring.

Keywords Tropical microalgae · Hexavalent chromium · Cadmium · Fluorescence · Growth media

Accumulation of chromium and cadmium in the aquatic environment by anthropogenic activities is a wildly recognized pollution issue. Chromium exists in the aquatic environment mainly in two oxidation states viz. Cr(III) and Cr(VI) of which the hexavalent form can easily cross biological membranes (Cervantes et al. 2001). Chromium presents a main environmental concern especially due to the effluents discharged by different types of industries such as chemical, steel and textile manufacturing, electroplating and leather tanning industries. Cadmium is persistent in nature and once absorbed by an organism, remains in the organism for a long period of time. Cadmium derives its toxicological properties from its chemical similarity to zinc forming Cd^{2+} ion. Cd is mostly used in Ni-Cd batteries, pigments, electroplating and as stabilizers for plastics (Adriano 2001). These metals are concentrated in water and tend to accumulate in bottom sediments from which they can be released by various processes of mobilization and can move up the biological chain, reaching humans and causing acute and chronic illnesses (Förstner and Wittmann 2012).

Microalgae play an important role in keeping the equilibrium of aquatic environments because they are the first level of tropic chain to produce organic matter and oxygen. Microalgae can be used for the environmental monitoring of pollutants such as heavy metals (Brayner et al. 2011). In environmental impact assessment studies based on ecotoxicological context, algal growth inhibition assays have been commonly used for establishing toxicity thresholds for sensitivity assessments. Micro-well plates are increasingly been used for algal growth inhibition assays which can reduce space and sample requirements (Eisentraeger et al. 2003). For the evaluation of the algal growth, direct parameters such as counting organisms under microscope as well as

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✉ I. V. N. Rathnayake
vayanga@kln.ac.lk

[1] Department of Microbiology, Faculty of Science, University of Kelaniya, Kelaniya, GQ 11600, Sri Lanka

[2] Department of Zoology and Environmental Management, Faculty of Science, University of Kelaniya, Kelaniya, GQ 11600, Sri Lanka

[3] Global Centre for Environmental Remediation (GCER), Faculty of Science and Information Technology, The University of Newcastle, University Drive, ATC Building, Callaghan, NSW 2308, Australia

indirect parameters such as absorbance and fluorescence measurements have been recommended (OECD 2011). Fluorescence measurements of microalgae are increasingly being used in recent times as it allows rapid processing of large number of samples (Mallic and Mohn 2003; Ferro et al. 2012). Microalgae contain the photosynthetic pigment chlorophyll which can absorb energy from light. During the photosynthesis process a small portion of energy absorbed as sunlight is emitted as fluorescence (Kumar et al. 2014). The amount of this fluorescence emission can be changed due to the inhibition of growth in the presence of contaminants like Cr^{6+} and Cd^{2+} in the environment. Therefore chlorophyll a fluorescence changes in microalgae can be used as an indicator for the monitoring and detection of heavy metals in the aquatic ecosystems.

However sensitivity of microalgae to these heavy metals may vary depending on the growth medium. Information on sensitivity of tropical algae to heavy metals such as Cr^{6+} and Cd^{2+} is meager in scientific literature. The objective of this study was to compare sensitivity of three tropical microalgae isolates to environmentally relevant concentrations of Cr^{6+} and Cd^{2+} in three types of algal growth media viz. BBM, TM1 and TM2 using fluorescence reduction as the proxy for growth inhibition. BBM is a popular growth medium used in the cultivation of microalgae and laboratory studies associated with microalgae (Bold and Wynne 1978). TM1 is a synthetic medium containing only the required major elements, devoid of chelators, iron and trace metals, used for the testing of maximum sensitivity (Peterson et al. 2005). TM2 medium is a synthetic reference media with low metal chelating capacity than BBM (Peterson et al. 2005). Except the presence of $NaNO_3$ in TM2 medium, the composition of the TM2 medium is very similar to the growth medium recommended by OECD (2011) for testing freshwater algae and cyanobacteria growth inhibition.

Materials and Methods

Three microalgae viz. *Mesotaenium* sp., *Chlorococcum* sp. and *Scenedesmus* sp. previously been isolated, from two freshwater ponds in Gampaha district, Sri Lanka which were identified using the morphological characteristics up to generic level according to Bellinger (1992) were used in this study. Periodically transferring to fresh media, axenic cultures of microalgal isolates were maintained in BBM medium, incubated at 25 ± 2 °C in flasks on the bench top orbital shaker (GFL® 3005) at 100 rpm (9.8 m/s^2), under continuous illumination (200 µE m^{-2} s^{-1} PPFD).

Stock solutions of Cr^{6+} and Cd^{2+} were prepared in deionized water using $K_2Cr_2O_7$ (≥ 99% purity, NORMAPUR, Belgium), and $Cd(NO_3)_2 \cdot 4H_2O$ (≥ 99% purity, Sigma-Aldrich, USA) respectively for toxicity assessments. Working solutions of Cr^{6+} and Cd^{2+} were prepared by appropriate dilutions of stock solutions. Metal solutions were sterilized and glassware was acid washed to avoid binding of metal to the glass surface. Microalgae sensitivity to two metal ions Cr^{6+} and Cd^{2+} were tested separately under three growth media viz. BBM, TM1 and TM2 using seven concentrations of each metal ion. Final nominal concentrations of the metal ion in the medium were 1, 2, 4, 8, 16, 33, 66 µg/L. The protocol of 'Algal microplate toxicity test suitable for heavy metals' (Peterson et al. 2005) was followed in the toxicity assessments. The composition of the BBM medium includes the stock1 (major stock) with $NaNO_3$, $CaCl_2 \cdot 2H_2O$, $MgSO_4.7H_2O$, K_2HPO_4, KH_2PO_4, and NaCl; the stock2 with EDTA and KOH; the stock3 with $FeSO_4$ and the stock4 (micronutrient stock) with trace metals H_3BO_3, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$ and $Co(NO_3)_2 \cdot 6H_2O$ (Bold and Wynne 1978). The TM1 medium had the stock 1 (macronutrients) including $NaNO_3$, NH_4Cl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, and KH_2PO_4 and the stock 4 including $NaHCO_3$ (Peterson et al. 2005). The TM2 medium contained the stock 1 (macronutrients) including $NaNO_3$, NH_4Cl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, and KH_2PO_4; the stock 2 (Fe chelator) including $FeCl_3 \cdot 6H_2O$, $Na_2EDTA \cdot 2H_2O$; the stock 3 (trace elements) including H_3BO_3, $MnCl_2 \cdot 4H_2O$, $ZnCl_2$, $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, and $Na_2MoO_4 \cdot 2H_2O$ and the stock 4 including $NaHCO_3$ (Peterson et al. 2005). The control consisted of only the growth medium. The protocol of 'Algal microplate toxicity test suitable for heavy metals' (Peterson et al. 2005) was followed in the toxicity assessments. Bioassays were conducted in quadruplicates in 96-well microplates (Sterilin®, flat bottom, sterile, with lid). Microalgal culture with a cell concentration of approximately 10^6 cells/mL (based on algal cell counts with a haemocytometer), corresponding growth medium (BBM or TM2 or TM1) and relevant heavy metal working solution were added to microplate wells. The growth control wells were also set using deionized water instead of the metal solution. The microplates containing microalgae suspension were incubated on an orbital shaker (GFL® 3005) at 100 rpm (9.8 m/s^2) under continuous illumination using cool white fluorescent lamps (200 µE m^{-2} s^{-1} PPFD). The Chlorophyll a fluorescence (using 440/40 nm excitation filter and 680/30 nm emission filter) at 24 h intervals from the time of initial inoculation up to 96 hours were measured using the Biotek Synergy™ HT Microplate Reader using Gen5 software. Relative fluorescence of microalgae as an indicator of growth was calculated as a percentage in relation to the untreated control for each tested concentration of each metal ion. Median effective

concentrations for fluorescence reduction (EC_{50}) were estimated by Probit analysis (Finney 1971) using MINITAB 15 Statistical Software™.

Visual MINTEQ (Version 3.1) was used to model the speciation of Cd and Cr in the different media compositions. The theoretical speciation of the heavy metals in three media was determined, along with the concentrations of free metals and predicted metal containing complexes and precipitates.

Analytical verification of the metal levels in the working solutions were analyzed by atomic absorption spectrometry [Analytik Jena model: novAA 400P] following standard analytical procedures (APHA 1999). The quality assurance and quality control components of this analysis consisted of duplicate analysis, five point calibrations with the standard metal solutions, reagent blank checks and reference standard checks. Limit of quantification (LOQ) for each of the analyte was estimated as the concentration that corresponds to the sum of the mean and ten times the standard deviation of 7 independent measurement of the blank medium (nitric acid). The estimated LOQ for Cr and Cd were 0.011 and 0.001 mg/L respectively. For the nominal concentrations of the metals in the working solutions (0.0125, 0.025, 0.05, 0.10, 0.20, 0.40 and 0.80 mg/L), the respective measured concentrations of metals were < 0.011 (LOQ), 0.023, 0.044, 0.08, 0.21, 0.42 and 0.89 for Cr and 0.013, 0.022, 0.045, 0.11, 0.21, 0.41, 0.76, mg/L for Cd. Since the nominal concentrations did not show much deviation from the measured concentrations of Cr and Cd in the working solutions, nominal concentrations were used in the analysis.

Results and Discussion

Information on the toxicity of tropical microalgae to environmentally relevant levels of heavy metals is rare in the scientific literature. The metal concentrations in the water from which the algae were isolated were very low. The pond water samples from which *Mesotaenium* sp. was isolated had 27 µg/L of Cr and 0.5 µg/L of Cd whereas Cr and Cd levels in the water samples from which the other two microalgae were isolated were 2 µg/L and 2.6 µg/L respectively (Munagamage et al. 2016).

The metal concentration–fluorescence reduction relationships (as % relative fluorescence) for the three microalgae grown in the three media at 24 h exposure are presented in Fig. 1 in Supplementary Material. Relative fluorescence (%) of each alga decreased with increasing metal ion concentration in each growth medium at 24 h of exposure indicating a concentration dependent graded toxicity response. Except for *Mesotaenium* sp., the concentration-response patterns were more or less similar in the growth media BBM and TM2. Concentration-response patterns of *Chlorococcum* sp. and *Scenedesmus* sp clearly indicate that the decrease in relative fluorescence (%) was relatively greater in the TM1 medium than in other two media (TM2 and BBM). Microalgae growth reduction at elevated chromium concentrations may be due to the hexavalent chromium toxicity. Cr(VI) can easily cross the algal cell membranes and inside the cells can convert to trivalent chromium by intracellular reduction. Intracellular Cr(III) can interact and affect the DNA causing

Table 1 Estimated 24 h EC_{50} values of Cr^{6+} and Cd^{2+} (for fluorescence decrease as a proxy for growth inhibition) for three microalgae grown in different types of culture media

Heavy metal	Culture medium	EC_{50} (µg/L)[a]		
		Mesotaenium sp.	*Chlorococcum* sp.	*Scenedesmus* sp.
Cr^{6+}	BBM	75 (67–86)	72 (63–83)	80 (69–95)
	TM1	52 (47–57)	54 (50–60)	57 (52–63)
	TM2	54 (45–67)	71 (64–80)	130 ([b])
Cd^{2+}	BBM	91 (78–112)	89 (76–110)	95 (81–118)
	TM1	66 (58–77)	68 (62–75)	70 (64–80)
	TM2	72 (59–95)	88 (77–106)	101 (83–133)

[a]95% confidence limits are given within parentheses

[b]Concentration range not sufficient to estimate confidence limits

Table 2 Estimated Cr and Cd metal speciation percentages (Visual MINTEQ) in culture media

Metal ion	Ion complex	Percentage in growth medium (%)		
		BBM	TM1	TM2
Cd^{2+}	Cd^{2+}	–	87.654	86.360
	$CdCl^{+}$	–	7.502	7.512
	$CdSO_4$ (aq)	–	1.381	1.394
	$CdHPO_4$ (aq)	–	1.388	1.394
	$CdHCO_3^{+}$	–	1.346	1.342
	$CdEDTA^{2}$	99.980	–	1.269
CrO_4^{2-}	CrO_4^{2-}	74.297	71.882	71.782
	$HCrO_4^{-}$	15.555	17.987	18.128
	$CaCrO_4$ (aq)	7.030	9.309	9.524
	$NaCrO_4^{-}$	2.445	0.813	0.557
	$KCrO_4^{-}$	1.095	–	–

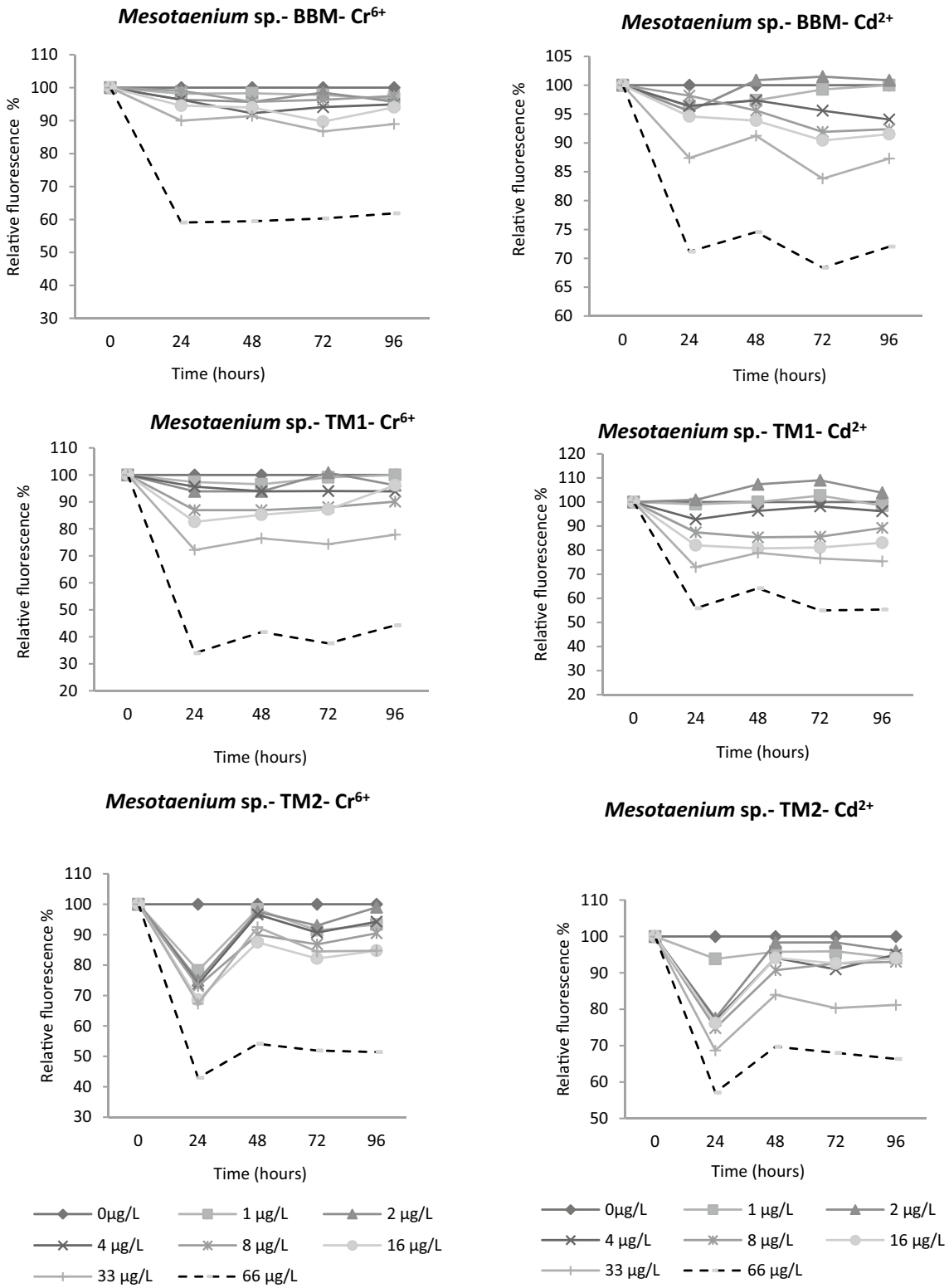

Fig. 1 Changes in the relative fluorescence patterns of microalgae *Mesotaenium* sp. in three different culture media; BBM, TM1, TM2 in the presence of heavy metal ions Cr^{6+} and Cd^{2+} with increase in the exposure time

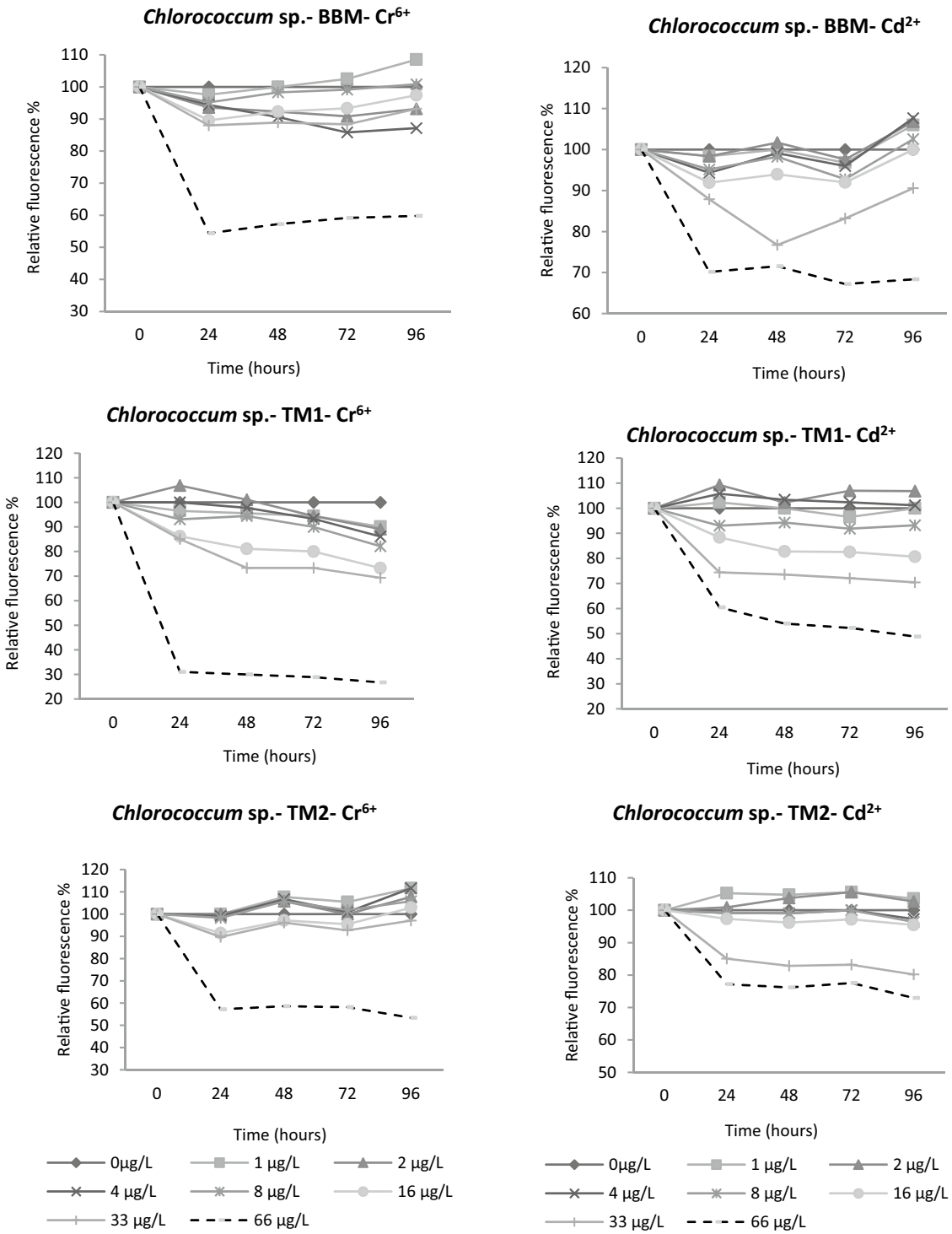

Fig. 2 Changes in the relative fluorescence patterns of microalgae *Chlorococcum* sp. in three different culture media; BBM, TM1, TM2 in the presence of heavy metal ions Cr^{6+} and Cd^{2+} with increase in exposure time

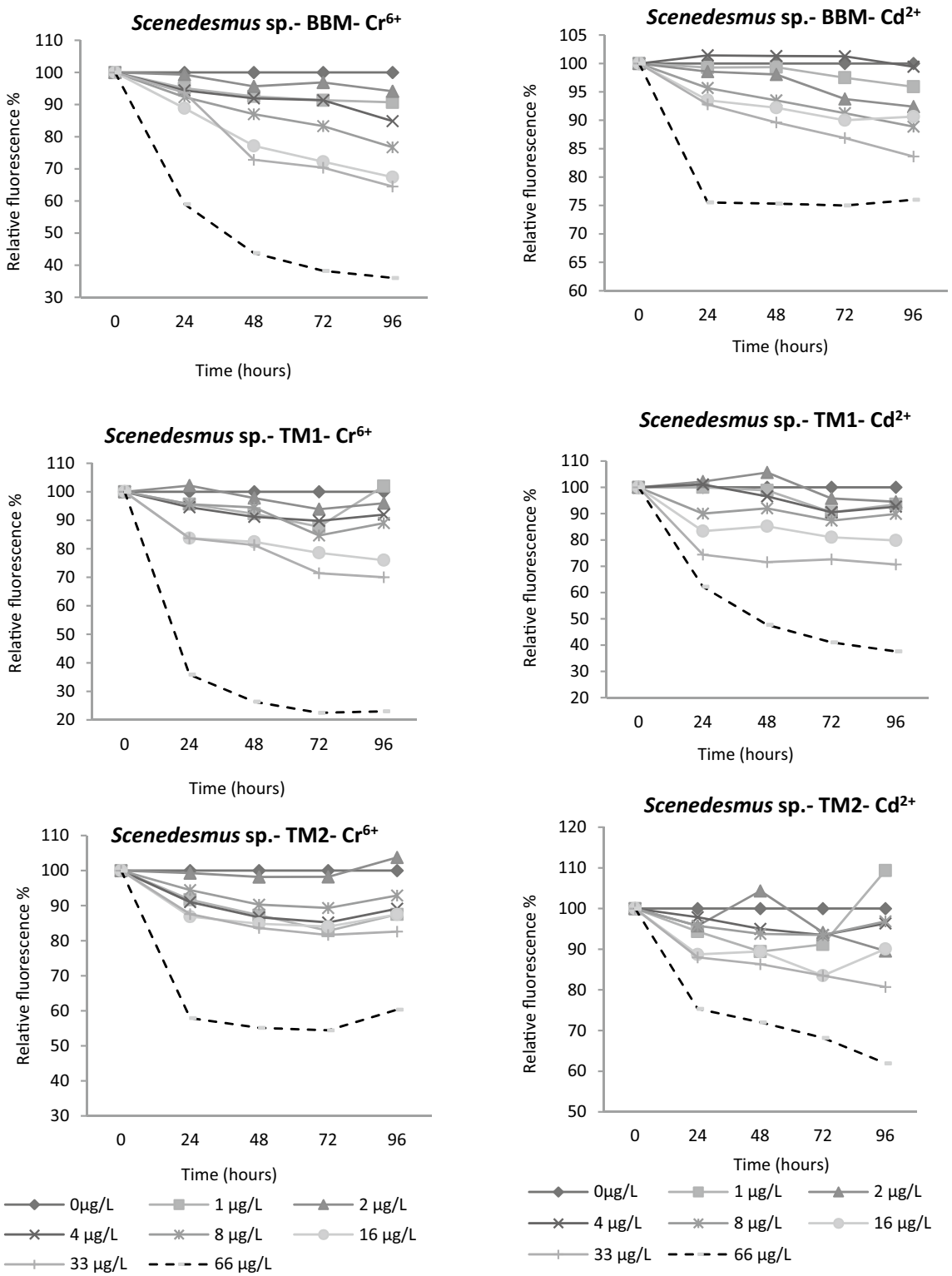

Fig. 3 Changes in the relative fluorescence patterns of microalgae *Scenedesmus* sp. in three different culture media; BBM, TM1, TM2 in the presence of heavy metal ions Cr^{6+} and Cd^{2+} with increase in exposure time

mutagenicity (Vignati et al. 2010). Chromium can interfere with the uptake of some essential elements such as Fe & S due to its structural similarity (Shankar et al. 2005). Inside algal cells, chromium stress can also result in alterations of photosynthetic pigments such as chlorophyll (Pereira et al. 2013). Chromium also produces reactive oxygen species that cause oxidative damage to cells and cellular mechanisms (da Costa et al. 2016). Cadmium also shows a high toxicity at elevated concentrations to microalgae. Cd is thought to have toxicity to photosystem II (PSII) by acting on the donor side or the acceptor side or inhibiting activity of oxygen evolving complex (Wang et al. 2013). Cd causes inhibition or inactivation of many enzymes mainly by its' binding to functional groups and thus shows inhibition of growth, photosynthesis or respiration in plant cells and algae. Hence, changes of chlorophyll fluorescence as observed in this study with Cr^{6+} and Cd^{2+} exposure may be due to inhibition of physiological processes in the algal cells which may indirectly indicate the inhibition of the growth. Table 1 presents the estimated 24 h median effective concentration (EC_{50}) of metal ions in three media, for the fluorescence reduction, as a proxy for growth inhibition. With respect to a specific metal ion exposure in a particular growth medium, no significant sensitivity differences were found among the three microalgae as the confidence limits for the EC_{50} values overlap with each other except for the *Scenedesmus* sp. grown in TM2 medium under Cr^{6+} stress where confidence limits could not be estimated. Of the three media tested, for both metal ions, the lowest 24 h EC_{50} values were found when all three microalga isolates were grown in the TM1 medium indicating that all microalgae were more sensitive to the metal ions when grown in the TM1. Moreover, comparison of EC_{50} estimates relevant to Cr^{6+} and Cd^{2+} showed that all microalgae were more sensitive to Cr^{6+} than Cd^{2+} when grown in the TM1 (Table 1). TM1 medium is a synthetic medium containing only the required major elements, devoid of chelators, iron and trace metals (Peterson et al. 2005). Although, the confidence limits of EC_{50} for *Mesotaenium* sp. grown in TM1 media overlap with the respective values obtained for the TM2, the algal cultures tested in TM1 medium showed the lowest EC_{50} values from all three media tested.

Visual MINTEQ (Version 3.1) metal speciation results (Table 2) indicate that both TM1 and TM2 media have higher percentage of freely available Cd^{2+} ions and Cd complexes such as $CdCl^{+}$, $CdCl_{2\,(aq)}$, $CdSO_{4\,(aq)}$ (Kituyi et al. 2017; Piotto et al. 2018; Liu et al. 2018) in the aqueous solution, and in BBM, majority of Cd (99.98%) was complexed with EDTA which greatly reduces the bioavailable Cd^{2+} content (Li et al. 2017). Majority of Cr was present as CrO_4^{2-} and $HCrO_4^{-}$ which are the typical mobile forms of Cr(VI) which is more toxic than Cr(III) (Kano 2018). Therefore greater toxicity (higher growth inhibition) of heavy metal ions in the TM1 medium could be attributed to the higher bioavailability of the heavy metal ions in the TM1 since it is a synthetic medium devoid of any metal chelating agents. According to Peterson et al. (2005), TM1 medium is formulated to detect maximum sensitivity of algae to heavy metals. BBM medium contains metal chelating agents such as EDTA which will bind the metal in the medium making it unavailable for the uptake by cells. This will reduce the bioavailability of heavy metal ions, therefore the microalgal cells are exposed to low amounts of Cr^{6+} and Cd^{2+} free ions than originally added amounts to the medium as shown in both metal speciation results and EC_{50} results.

The results obtained with BBM of the present study showed less sensitivity of *Mesotaenium s*p. to most of the higher concentrations used in comparison with the other two media used (Fig. 1 in Supplementary Material). Similar pattern was also reported earlier with the BBM growth medium (Juarez et al. 2008). Even though TM2 also contain EDTA as a metal chelating agent, the concentration of the component is reduced from the ISO level to concentrations sufficient to maintain log-phase growth for most test species for a 72-h period (Peterson et al. 2005). Although TM2 medium has less chelating capacity than BBM medium, overall EC_{50} results indicate no significant differences between TM2 and BBM with respect to the sensitivity of the microalgae as the corresponding confidence limits overlap with each other. The results suggest that the TM1 medium is more suitable under short term exposure for screening maximum sensitivity of freshwater microalgae to heavy metal ions in environmental monitoring and assessment studies. With the continuous exposure for 96 h, the sensitivity of the microalgae especially *Mesotaenium* sp. and *Chlorococcum* sp. to Cr^{6+} and Cd^{2+} was reduced in all three growth media (Figs. 1 and 2). *Scenedesmus* sp grown in TM2 and BBM media also displayed reduction in sensitivity to the highest concentration of Cr^{6+} and Cd^{2+} respectively with the 96 h exposure (Fig. 3). The growth response of *Mesotaenium* sp. and *Chlorococcum* sp. gradually reached near control levels in most of the low concentrations by the end of 96 h exposure. Less sensitivity of the microalgae to the metal ions may be due to the development of metal resistant mechanisms in the algal cells with the increase in exposure time.

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