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Whole cell microalgal-cyanobacterial array biosensor for monitoring Cd, Cr and Zn in aquatic systems

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ABSTRACT

Bioavailable content of metals in aquatic systems has become critical in assessing the toxic effect of metals accumulating in the environment. Considering the need for rapid measurements, an optical microalgal-cyanobacterial array biosensor was developed using two strains of microalgae, *Mesotaenium* sp. and a strain of cyanobacteria *Synechococcus* sp. to detect Cd^{2+}, Cr^{6+} and Zn^{2+} in aquatic systems. Microalgal and cyanobacterial cells were immobilized in a 96-well microplate using sol-gel method using silica. Optimum operational conditions for the biosensor array such as exposure time, storage stability, pH, and multiple metal effect were tested. A 10 min exposure time yielded optimum fluorescence values. Metal toxicity increased with decreasing pH, resulting in low relative fluorescence (%) and decreased with increasing pH, resulting in higher relative fluorescence (%). The optimum storage time for biosensor strains were 4 weeks for microalgal cultures and 8 weeks for cyanobacterial culture, at 4 °C storage temperature. The metal mixtures showed less effect on the inhibition of relative fluorescence (%) of microalgal/cyanobacterial cultures, displaying an antagonistic behavior among the metals tested. As a single unit, this photosynthetic array biosensor will be a valuable tool in detecting multi-metals in aquatic systems.

Key words: array, biosensor, cyanobacteria, fluorescence, heavy metals, microalgae

HIGHLIGHTS

- A whole-cell microalgal-cyanobacterial array biosensor was successfully developed for monitoring acute metal toxicity in water systems.
- Biosensor organisms respond to metals within 10 minutes' exposure.
- Survival potential of the biosensor organisms was higher at high pH.
- Antagonistic effect was shown by the biosensor organisms in metal mixtures.
- Biosensor was viable throughout 8 weeks of storage at 4 °C.

INTRODUCTION

Heavy metals can accumulate in the environment due to both natural and anthropogenic sources. The natural sources include weathering of parent materials such as rocks that contain high levels of heavy metals. The slow leaching of heavy metals from rocks and soils into the water by natural means does not cause much heavy metal pollution (Zhou *et al.* 2008). Heavy metal pollution is largely due to human activities such as purification of metals, including mining, smelting, ore refinement, preparation of nuclear fuels and other nuclear processing activities, industrial manufacture of batteries, electroplating, paints, pesticides, metal alloys, and many other products and agricultural practices. Heavy metal-rich effluents from these industries can contaminate water sources by adding runoff water to the downstream waterways by rain or by intentional discharge of wastewater by humans (Turdean 2011; Dey 2012; Islam *et al.* 2017). Heavy metal pollution of aquatic ecosystems including oceans, rivers, lakes and reservoirs is now a worldwide severe problem. This is mainly because, unlike organic pollutants, heavy metals do not decay because they cannot be degraded by usual biological, physical or chemical methods. The toxicity of heavy metals is inherent in their atomic structure and cannot be further mineralized into less harmful forms (Mohamed 2001; Wong *et al.* 2017). These heavy metals, especially metals such as arsenic, cadmium,

chromium, copper, nickel, lead, mercury and zinc, can bioaccumulate in aquatic organisms and may even cause toxicity to humans and other terrestrial animals via food chains (Islam *et al.* 2017). Considering the use of some lakes and rivers as water supplies, direct use of toxic metal-contaminated water for drinking, household and recreational purposes also poses a direct threat to human health (Zhou *et al.* 2008; Islam *et al.* 2017).

Therefore, it is essential to monitor and measure the metal content in aquatic environments. Chemical analysis of water and sediment is the most direct approach to reveal the heavy metal pollution status in the aquatic environment (Zhou *et al.* 2008). Although chemical methods are highly precise, accurate and can detect very low amounts of metals such as ppt (parts per trillion), they suffer from the disadvantages of high cost, the need for trained personnel to operate, and the fact that they are mostly laboratory bound (Verma & Singh 2005; Axelrod *et al.* 2016). Also, these methods are mainly used to measure the total metal content of an environmental sample rather than the bioavailable metal content, which gives a more accurate indication of potential risk to human and environmental health.

In order to fulfil this gap in analysis of biological effect of heavy metals on biological systems, 'biomonitoring' has become popular as a modern pollution analyzing technique. The most popular biomonitoring techniques include bioassays and biosensors. A biosensor is a device with the ability to biologically respond to an event, for example, the presence of a potentially harmful pollutant or cellular stress, and generate a detectable output signal, which is proportional to the event response (Lei *et al.* 2006). Detection mechanisms in biosensors can be based on surface-enhanced Raman scattering (SERS), fluorescence emission, electrochemical response (Dey 2011), etc.

The use of whole-cell microorganisms as biosensors has many advantages. Microorganisms are present ubiquitously and they have a short generation time, rapid response, ease of maintenance and storage, and low cost. They are able to metabolize a wide range of chemical compounds, have great capacity to adapt to adverse conditions and are amenable to genetic modifications through mutations or recombinant DNA technology (D'Souza 2001; Magrisso *et al.* 2008; Jiang *et al.* 2017). Types of whole-cell microbial biosensors include bacteria, yeast, microalgae and cyanobacteria.

From these microorganisms, eukaryotic microalgae (green algae) and prokaryotic cyanobacteria play an important role in keeping the equilibrium of aquatic environments because they are the first level of the trophic chain to produce organic matter and oxygen. Other aquatic biota are dependent on their photosynthetic activity. These microalgae and cyanobacteria are regarded as indicators for environmental monitoring and assessment because they are sensitive to many pollutants (Campanella *et al.* 2000; Gissi *et al.* 2015; Roxby *et al.* 2020).

The most preferred biological process to assess the effect of contaminants on microalgae is the photosynthetic activity, estimated by chlorophyll *a* fluorescence of photosystem II (PSII), or oxygen production (Altamirano *et al.* 2004). Algae and cyanobacteria are photosynthetic organisms that contain chlorophyll *a* pigments. During the photosynthesis process, a small portion of energy absorbed as sunlight is emitted as fluorescence (Wong *et al.* 2013a, 2013b). The amount of this fluorescence emission can be altered due to contaminants like heavy metals in the environment and their effect on algae and cyanobacterial photosystem II. Therefore, fluorescence changes in these organisms can be used as an indicator for the monitoring of environmental contaminants, especially heavy metals in the aquatic systems (Buonasera *et al.* 2011; Dudkowiak *et al.* 2011).

This study was focused on developing a whole-cell optical array biosensor using microalgae and cyanobacteria, which can be used in monitoring and risk assessment of aquatic systems for bioavailable heavy metals.

MATERIALS AND METHODS

Microalgal and cyanobacterial cultures

Two strains of *Mesotaenium* spp. and one strain of cyanobacteria, *Synechococcus* sp., isolated in previous studies (Munagamage *et al.* 2016, 2020) were used in this study. Axenic cultures of microalgal and cyanobacterial isolates were maintained in Bold's Basal medium and BG11 medium, respectively, by incubating at 25 $\pm$ 2 °C in flasks on a benchtop orbital shaker (GFL® 3005) (100 rpm), under continuous illumination (200 $\mu E\ m^{-2}\ s^{-1}$ PPFD). Cultures were periodically transferred to corresponding fresh media on a monthly basis.

Preparation of heavy metal solutions

Stock solutions of chromium as Cr^{6+} (100 mg/L, 10 mg/L), cadmium as Cd^{2+} (100 mg/L, 10 mg/L), and zinc as Zn^{2+} (100 mg/L, 25 mg/L) were prepared using $K_2Cr_2O_7$ (AnalaR NORMAPUR, Belgium), $Cd(NO_3)_2.4H_2O$ (Sigma Aldrich),

and $Zn(NO_3)_2.6H_2O$ (Sigma Aldrich) respectively. Using these stock solutions metal ion working solution series was prepared. For Cr^{6+} and Cd^{2+} the concentrations of working solutions were 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125 mg/L. For Zn^{2+} the concentrations of working solutions were 16.0, 8.0, 4.0, 2.0, 1.0, 0.5, and 0.25 mg/L. Concentrations of heavy metals in the working solutions were verified analytically using atomic absorption spectrometry (Analytik Jena model: novAA 400P atomic absorption spectrometer with a graphite furnace and autosampler or flame mode (Acetylene/air) where appropriate) as described in the standard analytical procedures (American Public Health Association (APHA) 1999). All metal ion solutions were sterilized by autoclaving. All glassware was acid washed before use to avoid binding of metal.

Immobilization of microorganisms

Sol-gel immobilization mixture was prepared by mixing equal volumes of 0.4 M sodium silicate (Sigma, USA) and 8.5 M colloidal silica (LUDOX AS-40, Aldrich) (Nguyen-Ngoc & Tran-Minh 2007a, 2007b; Ramachandran *et al.* 2009). The pH was adjusted to 7.5–8.0 using 4 M HCl. This mixture was used for the immobilization of algal/cyanobacterial cells. Aliquots of 20 μL of cell suspensions with a cell concentration of approximately 10^6 cells/mL of selected microalgal/cyanobacterial cultures (72 h old) were added to the wells of a 96-well microplate (Sterilin®, flat bottom, sterile, with lid) (Figure 1). Silica-gel mixture with pH adjusted to 7.5–8.0 (20 μL) was added on top of cell suspension in each well and mixed using the micropipette. The sol-gel/algal mixtures were allowed to solidify at the ambient temperature (25 °C).

Optimization of the biosensor

Effect of exposure time

Immobilization of selected algal/cyanobacterial cultures in the microplate was carried out as given above. A 180 μL volume of Test Medium 2 (TM2) (Peterson *et al.* 2005) was added to the top of the immobilized algal/cyanobacterial culture. Heavy metal ion solutions (20 μL each) (working concentrations 0.8, 0.7, 0.6, 0.4, 0.2, 0.1, 0.05 and 0 mg/L/control for Cr^{6+} & Cd^{2+} and 16.0, 12.0, 10.0, 8.0, 4.0, 2.0, 1.0 & 0 mg/L/control for Zn^{2+}) were also added. Fluorescence was measured (using 440/40 nm excitation filter and 680/30 nm emission filter) initially (0 min) and thereafter at 10 min intervals up to 150 minutes.

Effect of pH

The immobilization of selected algal/cyanobacterial cultures in the microplate was carried out according to the procedure indicated above. Aliquots of 180 μL of TM2 medium were added to the top of the immobilized algal/cyanobacterial mixture, and 20 μL of each of the heavy metal ion solutions (working concentrations 0.8, 0.6, 0.4, 0.1 and 0 mg/L/control for Cr^{6+} and Cd^{2+} and 32.0, 16.0, 8.0, 2.0 and 0 mg/L/control for Zn^{2+}) adjusted to different pH (5, 6, 7, 8, and 9) was also added according to the plate layout. Fluorescence was measured using the Biotek Synergy™ HT Microplate Reader using Gen5 software initially (0 min) and thereafter at 10 min intervals up to 150 minutes.

Optimization of the storage stability of the biosensor

One microplate containing immobilized algal/cyanobacterial cells was taken and 180 μL of TM2 medium was added on top of the immobilized algal/cyanobacterial mixture. Then, 20 μL of selected heavy metal concentration was added to each microplate well of the selected plate according to the plate layout. Fluorescence was measured immediately, and the metal–medium mixture was drawn out using a micropipette leaving only the immobilized cells in silica gel-algal matrix. The wells were washed with sterilized distilled water and the microplate was stored at 4 °C in a refrigerator along with other prepared microplates. After 1 week of storage, the first microplate and a fresh microplate were taken and wells were filled with TM2 medium and kept for 30 minutes for the cells to revive. Then the heavy metal solution was added as

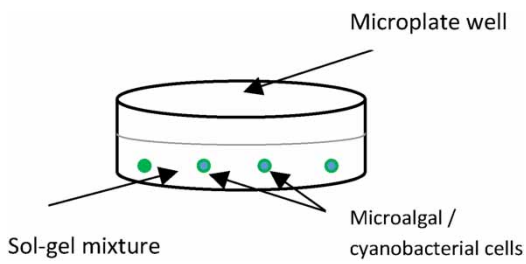

Figure 1 | Schematic diagram of the immobilization of the microalgal/cyanobacterial cells in the microplate well.

stated above. The fluorescence was measured in both plates; metal–medium mixture was drawn out, and the plates were washed with distilled water and stored at 4 °C. On the third week, the first microplate and another fresh microplate were taken and the fluorescence measuring procedure was repeated before putting to storage again. This was repeated for 8 weeks by repeatedly using the first-week microplate and using another fresh microplate each week.

Effect of the multiple metal mixtures on biosensor organisms

The immobilization of selected algal/cyanobacterial cultures in the microplate was carried out according to the procedure indicated above. TM2 medium (180 μL) was added on top of the immobilized algal/cyanobacterial mixture. Metal solutions based on tolerance limit of the specific metal in the industrial effluent discharged into inland surface waters (0.1 mg/L for Cr^{6+} and Cd^{2+}, 2.0 mg/L for Zn^{2+}) were mixed (20 μL) to make single metal solutions, binary mixtures (Cr + Cd, Cr + Zn, Cd + Zn) and tertiary mixtures (Cr + Cd + Zn) according to the plate layout in each well. Fluorescence was measured initially (0 min) and thereafter at 10 min intervals from mixing, up to 150 minutes.

Data analysis

Percentage survival based on fluorescence (relative fluorescence %) was calculated in relation to the untreated control for each tested concentration of each metal. Effective Concentrations (EC_x) x = 50 (estimated metal concentration value where the organism shows the relevant % reduction in survival compared to the control) were calculated using the Probit analysis (Finney 1971), using MINITAB 15 Statistical Software™. EC values are presented as mean values (±standard error), along with the 95% confidence intervals. The significant differences between fluorescence responses were calculated by analysis of variance (ANOVA), Tukey's test using MINITAB 15 Statistical Software™. The data were considered significant if $p \leq 0.05$.

RESULTS AND DISCUSSION

Immobilization of biosensor organisms in a sol-gel matrix

Effects of pH on solidification time of the sol-gel immobilized microalgae and cyanobacteria are presented in Table 1.

According to the results obtained, the ideal pH range for the solidification of the mixture in a short period of time was taken as pH 7.0–8.5.

Determination of the optimum exposure time of the biosensor

The selected microalgal/cyanobacterial cultures were exposed to the relevant metal ion, and fluorescence was measured and growth percentage calculated at 10 min intervals. The optimum exposure time was taken when the cultures showed the lowest growth drop from the initial time of measurement (Figure 2). The concentration-response relationships of the biosensors at 10 minutes' exposure to metals are shown in Figure 3. The percentage survival of biosensors decreased with increasing metal concentration (Figure 3). All the cultures showed 10 minutes of exposure to the metal ion as the shortest time to respond to metal toxicity (Table 2).

Determination of the effect of pH on the biosensor organisms

The fluorescence response relationships of biosensor organisms at different pH values to different concentrations of relevant heavy metals are shown in Figures 4 and 5.

Table 1 | Effect of pH on solidification time of the silica gel mixture

Na-silicate volume (mL)	LUDOX volume (mL)	pH value	Time required for solidification (min)
5	5	9.0	No solidification
5	5	8.5	20
5	5	8.0	15
5	5	7.5	10
5	5	7.0	20
5	5	6.5	No solidification

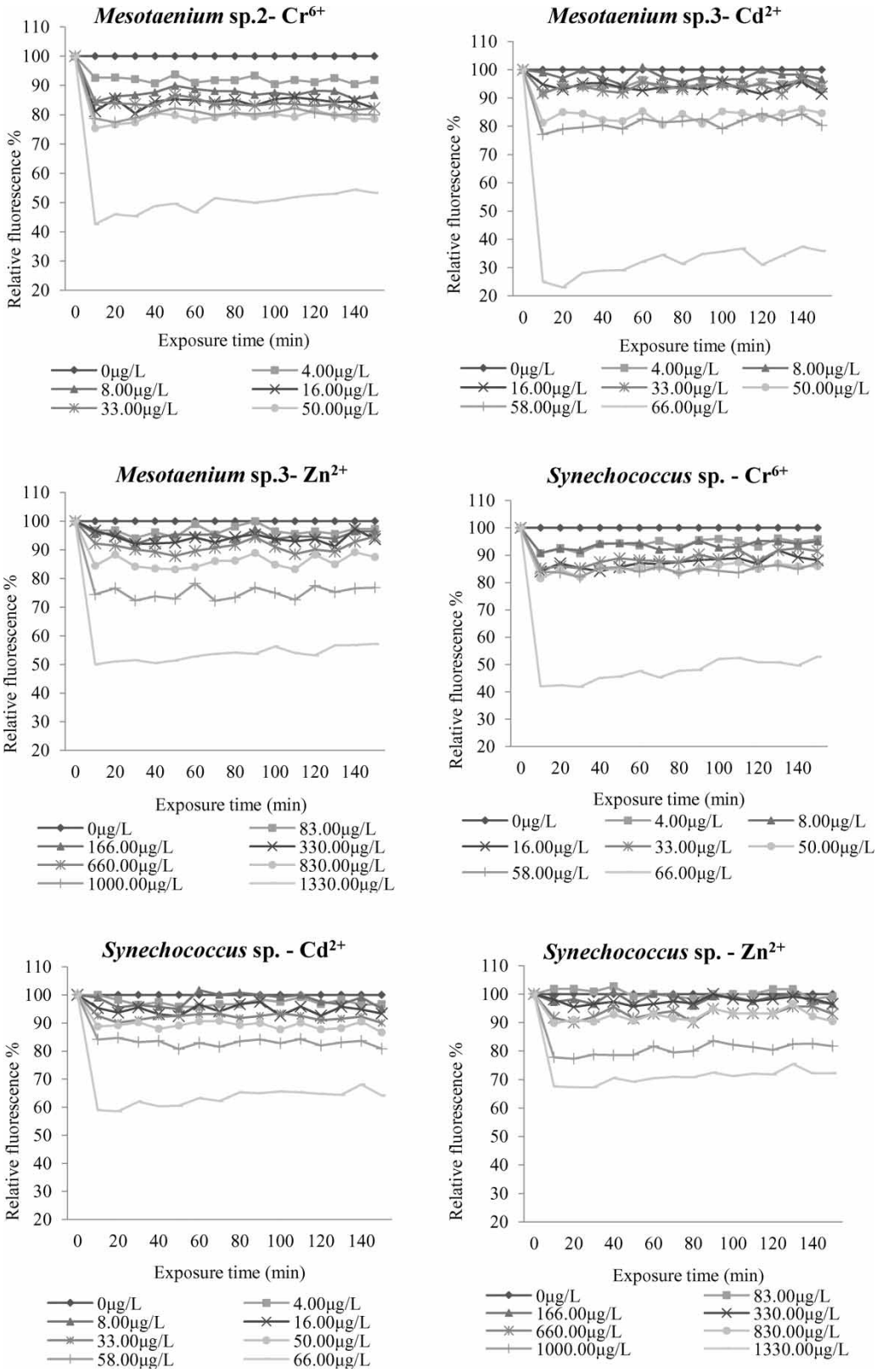

Figure 2 | Percentage survival relationships of biosensor organisms with exposure time, for relevant metal ions.

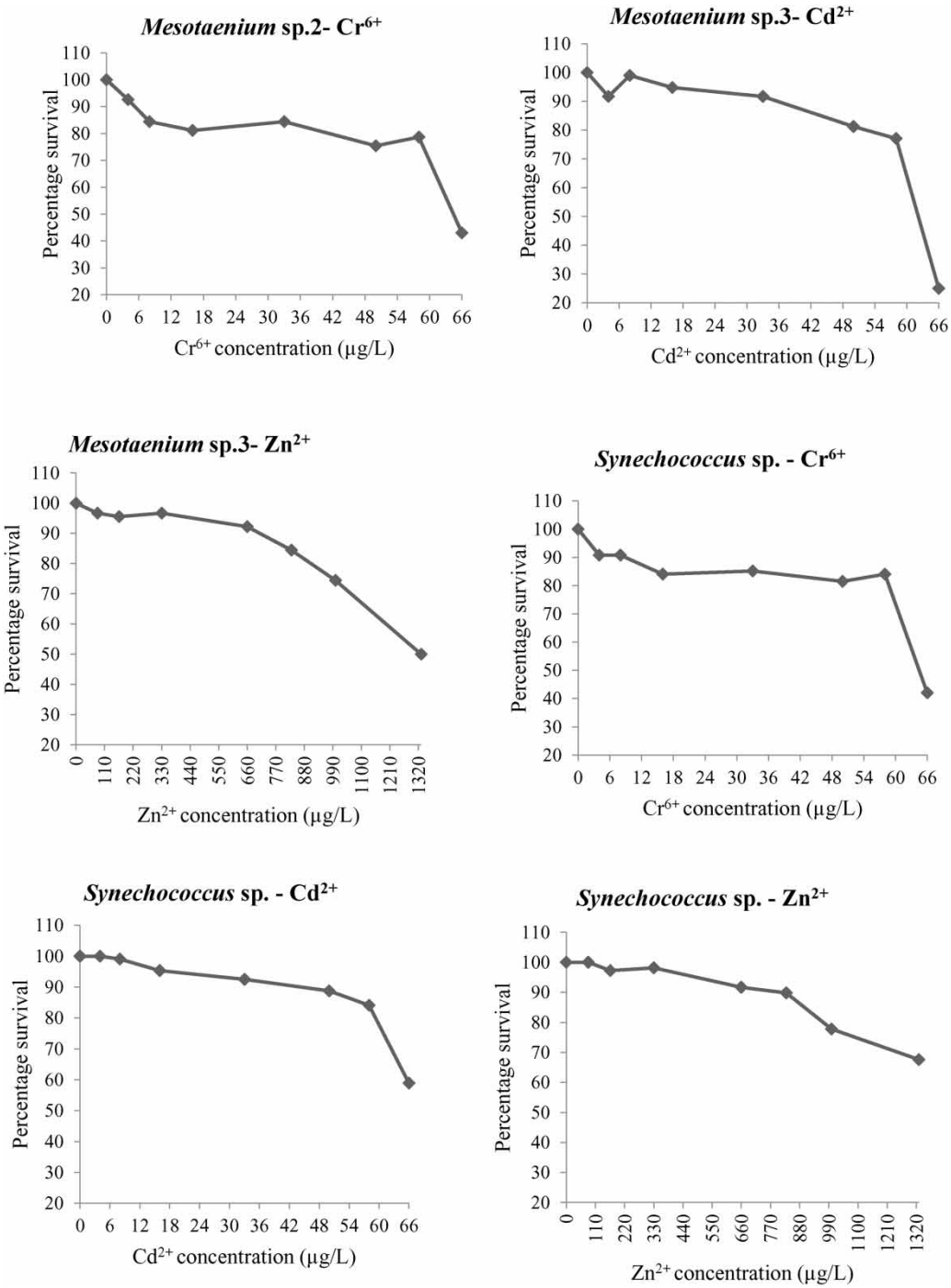

Figure 3 | Concentration-response relationships of the biosensor organisms at 10 minutes (optimum exposure time).

The biosensor organisms showed no significant differences in the fluorescence values at different pH values (5, 6, 7, 8, 9) when tested at low metal concentrations (Figures 4 and 5). However, at higher metal concentrations, there were significant differences in the fluorescence at different pH values.

Effect of multiple metal ions on the biosensor

Response of the biosensor to single metal (metal ion concentrations: 8.0 µg/L for Cr and Cd; 166 µg/L for Zn), and multiple metals were investigated by comparing the fluorescence relationships (Figure 5) and survival patterns (Figure 6) of the biosensor.

Table 2 | Optimum exposure time values for selected cultures

Algal/cyanobacterial culture	Tested metal ion	Optimum exposure time (min)	EC_{50} (μg/L)
Mesotaenium sp. 2	Cr^{6+}	10	77.2 (±5.3) (68.4–90.2)
Mesotaenium sp. 3	Cd^{2+}	10	65.9 (±2.4) (61.6–71.4)
Mesotaenium sp. 3	Zn^{2+}	10	1,377 (±62) (1,270–1,521)
Synechococcus sp.	Cr^{6+}	10	80.9 (±5.5) (71.7–94.7)
Synechococcus sp.	Cd^{2+}	10	81.0 (±4.2) (74.0–91.4)
Synechococcus sp.	Zn^{2+}	10	1,588 (±91) (1,436–1,814)

According to Figure 7, there were significant differences between the individual metal ions (Cr, Cd, and Zn), but the significance between binary and tertiary metal mixtures was low.

Both microalgal (*Mesotaenium* sp. 2 and *Mesotaenium* sp. 3) and cyanobacteria (*Synechococcus* sp.) cultures in the biosensor array displayed a greater inhibition of growth with individual metal ions (Cr^{6+}, Cd^{2+}, Zn^{2+}) than with a mixture of metal ions (Figure 6). The most substantial effect was for Cr^{6+} individual ion on all the cultures. Individual Zn^{2+} ion effect was lower than that of Cr^{6+} and Cd^{2+} on all three cultures. In all three cultures, combined metal effect was lower than the single metal effect. The lowest growth inhibition effects were shown by metal combinations Cd + Zn and Cr + Cd + Zn in all cultures.

Hexavalent chromium is highly toxic to microorganisms and plants. The diverse Cr-resistance mechanisms displayed by microorganisms include biosorption, diminished accumulation, precipitation, reduction of Cr(VI) to Cr(III), and chromate efflux (Cervantes *et al.* 2001). It is quite likely that the microorganisms in this study have shown some of these mechanisms of Cr bioremediation, leading to significantly lower fluorescence than the other single or mixed metal species.

Determination of the storage stability of the biosensor

Fluorescence responses of the biosensor organisms in the absence of heavy metals (control) with storage time are shown in Figure 8.

Figure 8 shows that the cells remained viable throughout the 8-week test period, giving a noticeable fluorescence value each week. However, the microalgal cultures *Mesotaenium* sp. 2 and *Mesotaenium* sp. 3 did not show any significant difference between the fluorescence values in the first 4 weeks while a significant difference was observed in the 5th week. The cyanobacterial culture *Synechococcus* sp. showed no significant difference in all 8 weeks of storage time.

Although most biosensors are developed using a single algal or cyanobacterial species, it is more beneficial to develop an array biosensor consisting of both prokaryotic cyanobacteria and eukaryotic microalgae, in the sense that it can be used to detect a wide range of pollutants (Podola & Melkonian 2005). Highly selective biosensor systems measure compounds with a high specificity and thus a further identification of the compound is not required. However, considering the large number of potentially hazardous substances occurring in the environment, high selectivity may also be regarded as disadvantageous. Combining the advantages of the two groups of biosensors, a non-selective biosensor allowing the identification of the compounds would be favourable (Podola *et al.* 2004).

Immobilization of biosensors using silica gel matrices is one of the current popular immobilization methods (Moretta *et al.* 2021). The cells of microalgae and cyanobacteria cultures selected from the dose-response assessment were immobilized in a semi-solid matrix using the silica sol-gel method. A mixture of colloidal silica and sodium silicate was prepared and the optimum pH value for the efficient solidification was tested by changing the pH value of the mixture. The use of equal volumes of sodium silicate (0.4 M) and LUDOX (8.5 M) for the immobilization was carried out according to Nguyen-Ngoc & Tran-Minh (2007a, 2007b) and Ramachandran *et al.* (2009). The ideal pH for the solidification of mixture in the shortest period of time was found to be between 7.5 and 8.5. Nguyen-Ngoc & Tran-Minh (2007b) studied the effect of cell encapsulation pH on the gelation time in the silica matrix for pH ranging from 6.0 to 8.5. According to their study, the minimum gelation time was observed at pH 7.0–7.5. We observed no gel formation beyond pH 8.5 because the particles are so charged that they

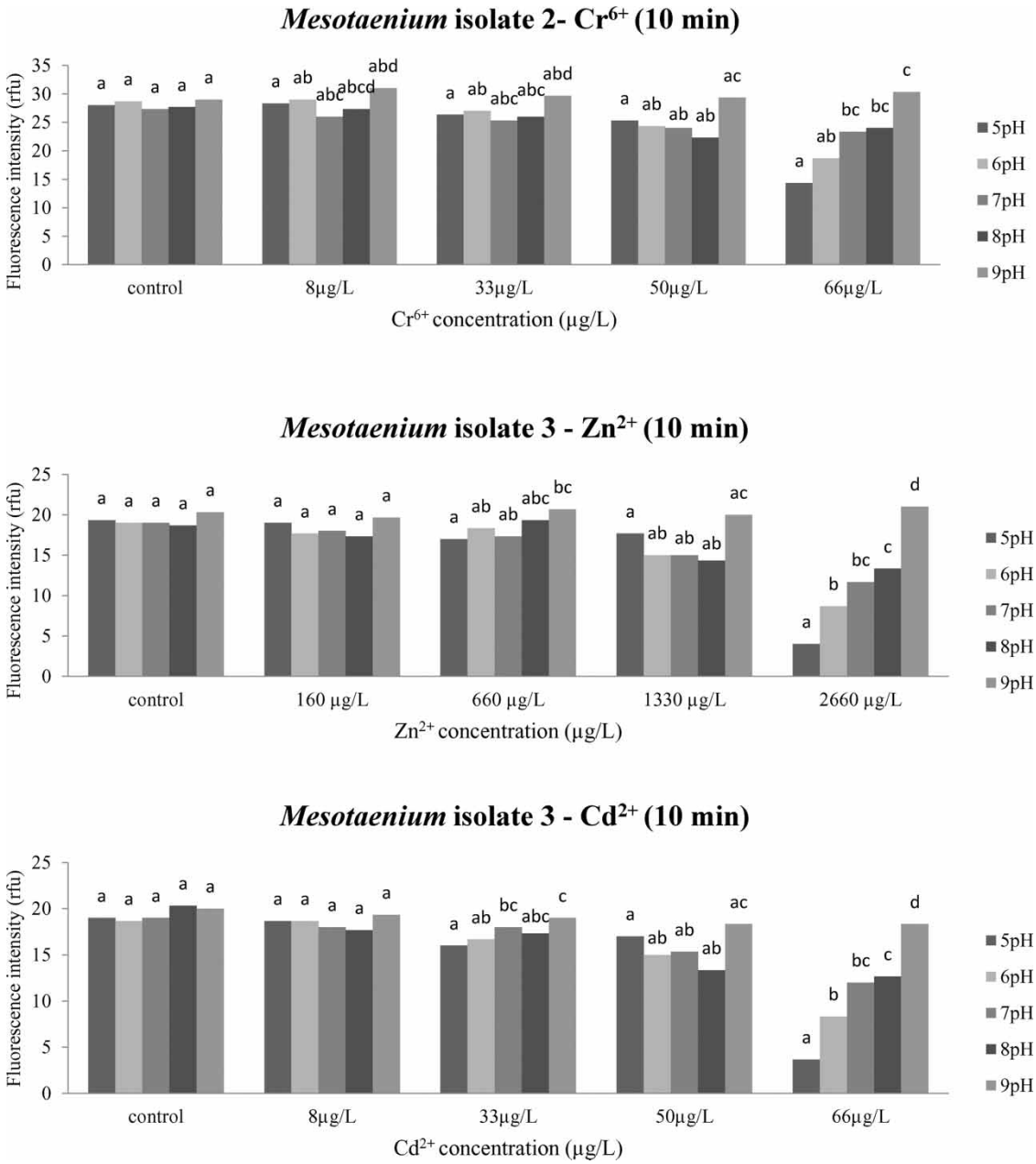

Figure 4 | Fluorescence response relationships with pH of the biosensor organisms at different metal concentrations. For a specific concentration, data shown with different letters are significantly different from each other (ANOVA, $p < 0.05$, Tukey's Test).

cannot agglomerate to undergo the polymerization process (Nguyen-Ngoc & Tran-Minh 2007b). A sol-gel solution undergoes sequential hydrolysis, polycondensation and dehydration (Dey & Naughton 2019; Dey 2020). There are two types of sol-gel precursors: alkoxide and silicate (Dickson & Ely 2013). Silicate precursors show osmotic/ionic stress related to salt content and larger fluctuation in gel stability with pH. So pH control is crucial for silicate precursor-based sol-gel systems. The first pKa of silicic acid occurs at pH 9.8, so it is fully protonated at acidic pH, suppressing condensation. This explains why acidic pH did not show any solidification. An equal volume of algal culture and sol-gel mix was added to the microplate wells during the immobilization process. This may vary in other literature. For example, in a study carried out by Soltmann *et al.* (2010) on algae-silica hybrid materials for biosorption of heavy metals, the algae content used in immobilization was 20% (wt/wt SiO_2). In some cases, algae content up to 50% was used. But in contrast to these results, in the study by Nguyen-Ngoc & Tran-Minh (2007b), it was shown that the amount of immobilized algal cells also affects the gelation process.

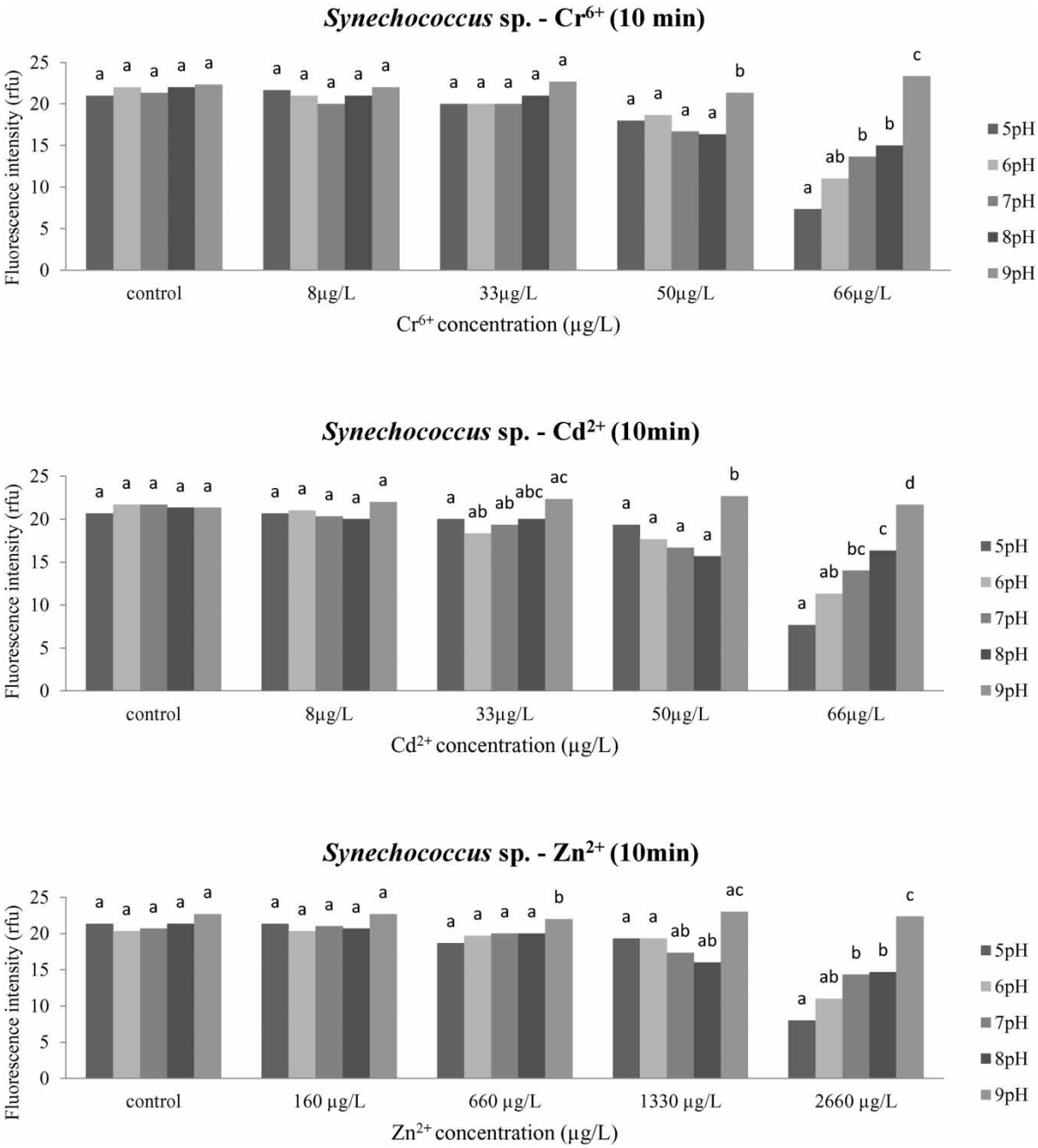

Figure 5 | Fluorescence response relationships with pH of the biosensor organisms at different metal concentrations. For a specific concentration, data shown with different letters are significantly different from each other (ANOVA, $p < 0.05$, Tukey's Test).

The microalgal and cyanobacterial cultures were chosen based on their sensitivity to the selected heavy metals (based on dose-response, EC_{50} values) from our previous studies (Munagamage *et al.* 2016, 2020) and were immobilized in a single microplate and exposed to metal ions. From calculating the growth percentage with time it was observed that the greatest drop in growth was observed when the cultures were exposed to metals for 10 min (Figure 2). Therefore, highest sensitivity was observed in all cultures tested at 10-minute time period. This value is taken as the optimum value to expose the prepared biosensor to environmental contaminants. A study on developing a biosensor to detect heavy metals and pesticides using cyanobacteria *Anabaena torulosa* by Wong *et al.* (2013b) reported that the optimum exposure time for the toxicants considered was 30 minutes. Studies on biosensors for heavy metals by Chouteau *et al.* (2004, 2005) using microalga *Chlorella vulgaris* gave

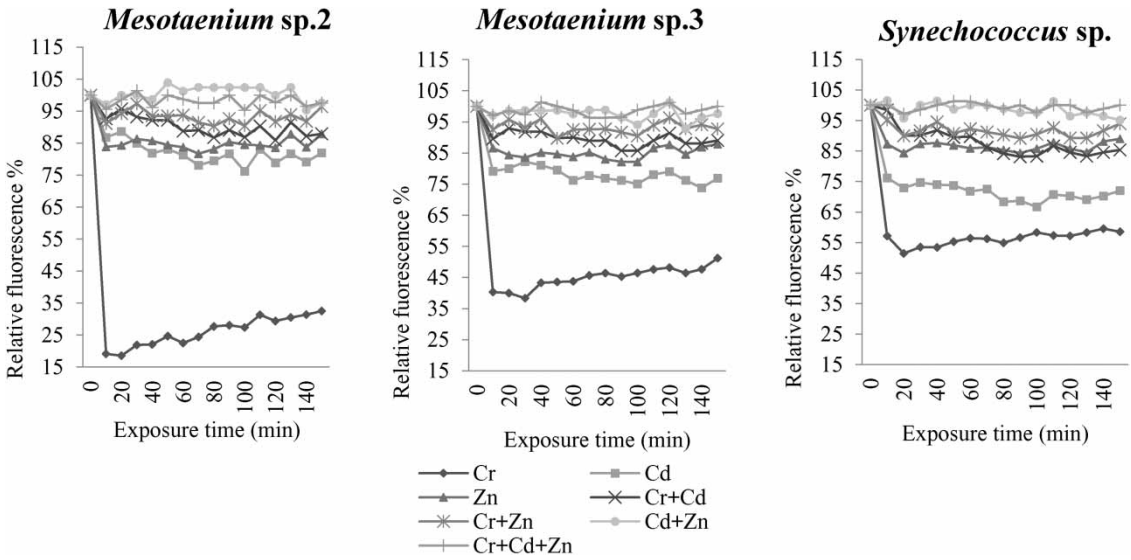

Figure 6 | Effect of individual and multiple metal ions on the biosensor organisms.

the optimum exposure time as 20 minutes and 30 minutes. A study by Durrieu *et al.* (2004) using whole-cell algal biosensors to detect toxic pollutants, including heavy metals, also used exposure times of 20 and 30 minutes. In the study on the development of a cyanobacterial biosensor by Shao *et al.* (2002) for herbicides, it was concluded that an assay period of 30 minutes was suitable for detection of most of the herbicides studied. In that study, the biosensor was also tested for non-herbicide toxicants such as the heavy metals copper and zinc, where the EC_{50} values were obtained at 15 minutes of exposure.

Detection of metals by the algal/cyanobacterial biosensor is explained in terms of the reduction in chlorophyll fluorescence in the presence of the metals in their bioavailable form. As reported by Appenroth (2010), these bioavailable metals are known to affect the light and dark reactions, structure of the chloroplast membrane, light-harvesting and oxygen-evolving complexes, photosystems, and the photosynthetic electron transport chain elements, and also inhibit the reductive pentose phosphate cycle. Many studies have shown the toxic effects of metals at higher concentrations on several algae through affecting the photochemistry of PSII system, detrimental impact on chloroplast structure or altering biochemicals such as proteins and lipids (Kumar *et al.* 2014).

In the present study, the biosensor organisms showed no significant differences in the fluorescence values at different pH values ranging between 5 and 9 when exposed to low metal concentrations. However, at higher metal concentrations, there were significant differences in the fluorescence at different pH values. This could be due to the inhibitory effect of metals on photosynthesis of the biosensors. In general, the low pH enhances the solubility of metals, thereby increasing their bioavailability and toxicity. The toxicity of metal ions to the tested algal species increased with the decrease of pH due to presence of metals in their free ionic state at low pH (acidic) conditions (Rai *et al.* 1993; Arunakumara & Zhang 2008; Maier *et al.* 2009). Changes in the pH also may affect the biological sensitivity by conformational changes in the metal-binding sites at the cell surface (Starodub *et al.* 1987). At lower metal concentrations there were only slight deviations in the toxicity. On the other hand, most cultures showed higher survival at higher pH and lower survival at lower pH, especially at higher metal concentrations. The pH of water is an important factor affecting the toxicity of metals to freshwater microalgae, although the relationship between pH and toxicity is largely algal species-dependent (Wilde *et al.* 2006). Many studies have been carried out on the effect of pH on the heavy metal toxicity to aquatic biota. A study by Starodub *et al.* (1987), on the effect of complexation and pH on heavy metal (Cu, Zn, Pb) toxicity to a freshwater green alga, *Scenedesmus quadricauda*, revealed enhanced toxicity at acidic pH. The combined toxicity of these metals was significantly greater at pH 4.5 than at pH 8.5 or pH 6.5. In a study by Rai *et al.* (1993), where an acid-tolerant strain and a wild-type strain of *Chlorella vulgaris* were exposed to Cu at varied pH (pH 6.8, 5.0, 4.0 and 3.5), a general reduction in growth, NH^{4+} uptake, photosynthesis and nitrate reductase activity of both the strains was noticed at decreasing pH.

In contrast, other studies have shown an increase in metal toxicity with increasing pH, which may occur as a result of decreased competition between the metal ion and H^{+} at the cell surface (Wilde *et al.* 2006), where Macfie *et al.* (1994) and Franklin *et al.* (2000) have shown similar results.

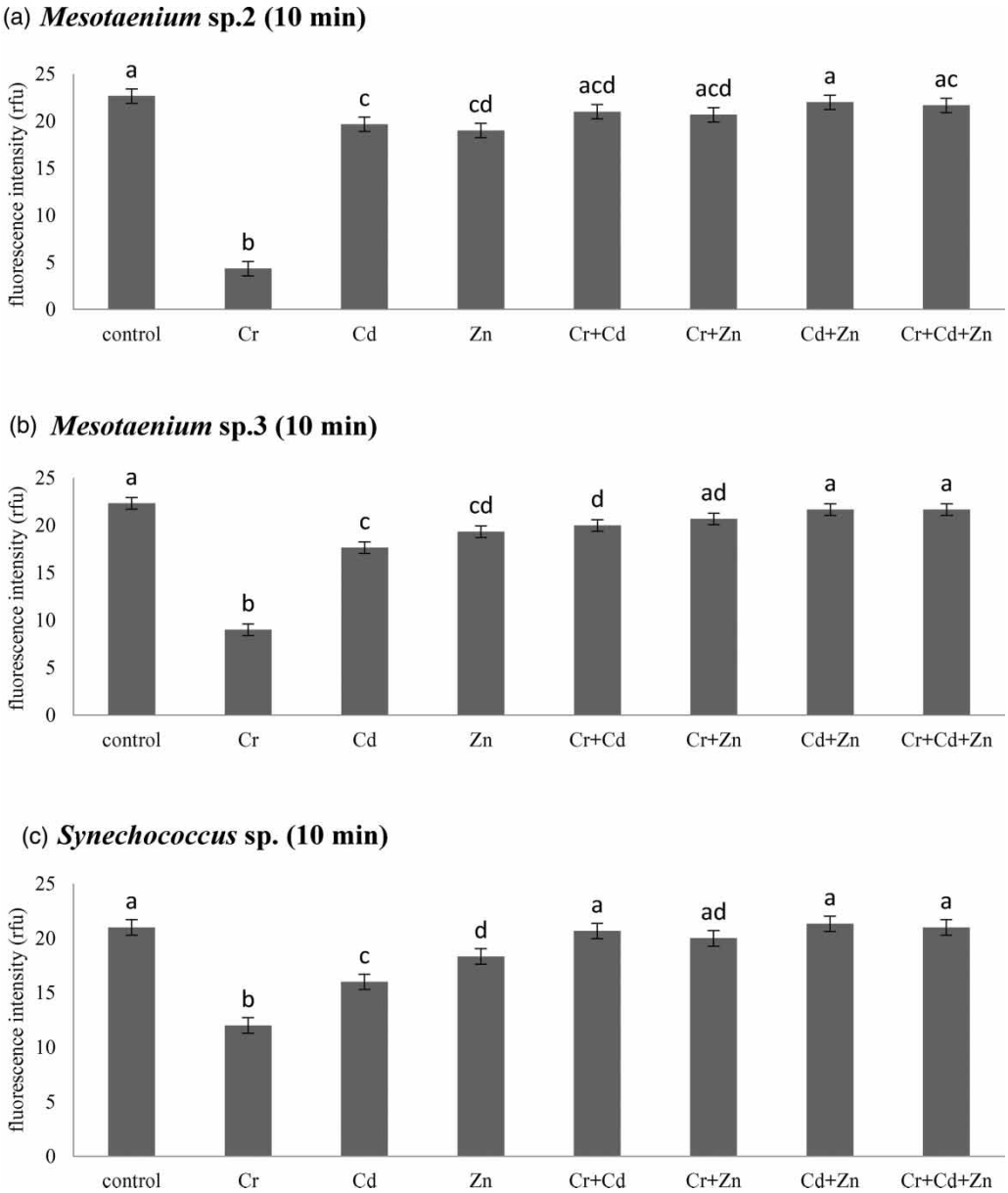

Figure 7 | Fluorescence relationships of biosensor organisms (a), (b), (c) to different metal ion combinations relevant to 1:12 dilutions of water effluent tolerance limits (8.0 μg/L for Cr and Cd; 166 μg/L for Zn) (Board of Investment 2011). Bars indicated with different letters for a specific organism are significantly different from each other (ANOVA, $p < 0.05$, Tukey's Test).

The microalgal and cyanobacterial cultures of the biosensor array were tested for both individual metal ions (Cr^{6+}, Cd^{2+}, Zn^{2+}) and a combination of metals (binary mixtures Cr + Cd, Cr + Zn, Cd + Zn, and tertiary mixture Cr + Cd + Zn). The metal ion combinations at 1:12 dilution with water effluent (Board of Investment 2011) were used in this experiment rather than the 1:8 dilution generally used to observe the effect of metal ion mixtures at a lower concentration level. As shown in Figure 7, there were remarkable significant differences between the individual metal ions (Cr^{6+}, Cd^{2+}, and Zn^{2+}), but the significant difference between binary and tertiary metal mixtures was low. All three cultures showed greater inhibition of growth when tested with individual metals, while the toxic effects of the metal combinations were lower. In general, a mixture of heavy metals can produce three possible types of effects; synergistic, antagonistic or non-interactive. Synergism is the phenomenon where the effect of the mixture is greater than that of each of the individual effects of the constituents of the mixture. Antagonism is where the effect of the mixture is less than that of each of the individual effects of the constituents of the mixture. Non-interaction is where the effect of the mixture is no more or no less than that of each of the

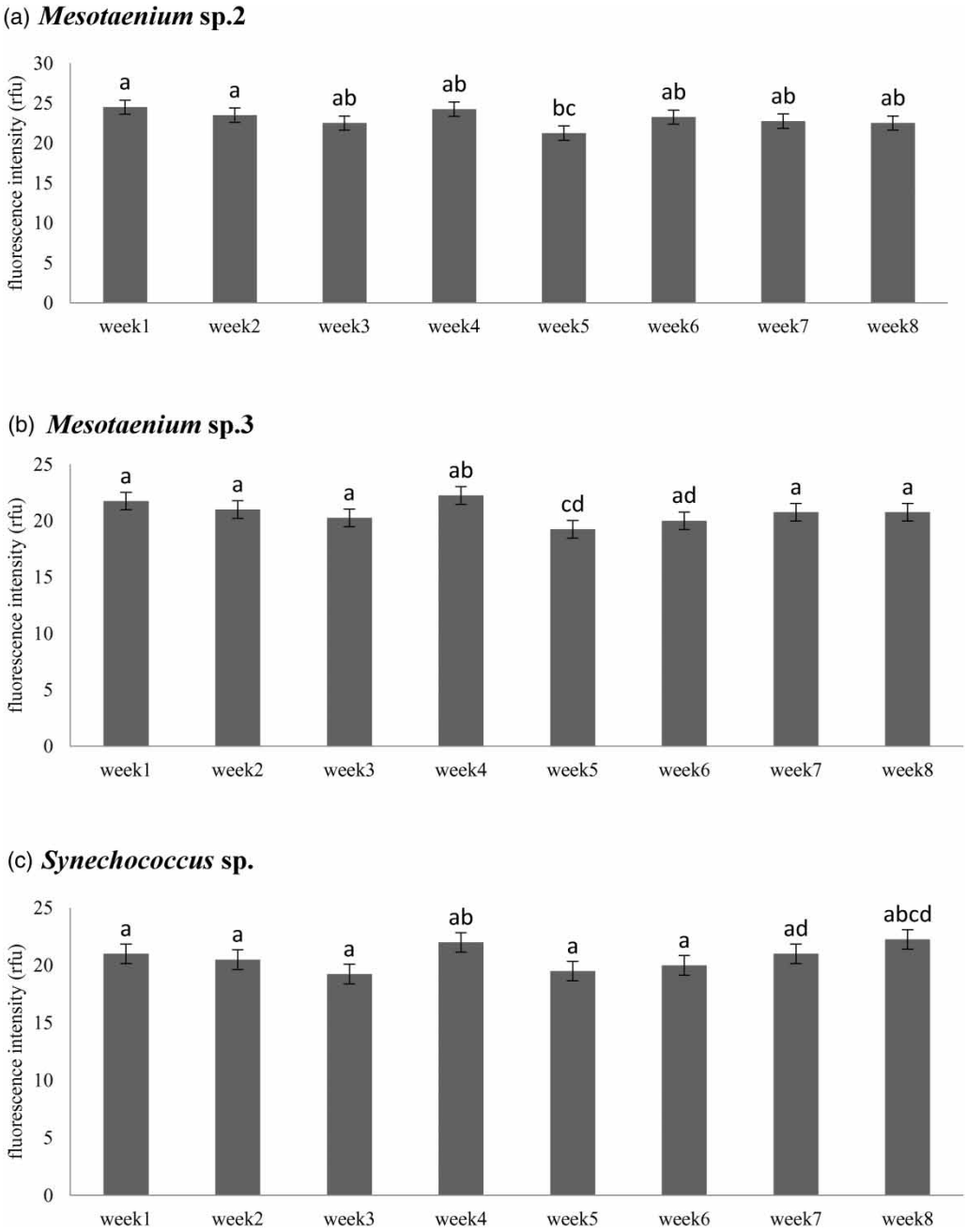

Figure 8 | Fluorescence relationships of biosensor organisms (a), (b), and (c) at different periods of storage. Bars indicated with different letters for a specific organism are significantly different from each other (ANOVA, $p < 0.05$, Tukey's test).

individual effects of the constituents of the mixture (Ting *et al.* 1991). In this study, the metal mixtures showed less effect on the inhibition of growth of microalgal/cyanobacterial cultures. Therefore, it is possible that the metal mixtures display an antagonistic behavior. This antagonistic effect is very prominent between the metal mixtures Cd + Zn and Cr + Cd + Zn. According to Sunda & Huntsman (1996, 1998) and Wei *et al.* (2003), the antagonism observed between Cd and Zn was more likely due to competition between metals for the uptake sites. According to Sunda & Huntsman (1996, 1998), concerning the coastal diatom *Thalassiosira pseudonana*, the Cd enters the cells through either Zn or Mn ion channels. Therefore, the presence of high free Zn^{2+} or Mn^{2+} can decrease Cd uptake and ultimately, its toxicity. Franklin *et al.* (2002) also observed an antagonistic behavior between metal combinations Cu + Zn, Cd + Zn and Cu + Cd + Zn in *Chlorella* sp. Although studies

conducted on Cr in a multiple metal mixture are rare, it is possible that the antagonistic effect between Cd and Zn also affect Cr toxicity.

Since the biosensor was prepared in the laboratory, before using it for environmental applications, the biosensor should be checked for its storage stability. The array biosensor consists of all three algal/cyanobacterial cultures, each selected for sensitivity to the metal ions tested (Cr^{6+}, Cd^{2+}, Zn^{2+}), immobilized in a single microplate. This microplate was stored at 4 °C in a sterile condition, and each week the immobilized cultures were revived by adding the growth medium on top of the culture-silica gel mixture. Then the metal solution was added to the plate and fluorescence was measured on a weekly basis. The fluorescence measurement was carried out for a single microplate repeatedly stored and tested each week and also for a set of eight fresh microplates with immobilized cultures stored at 4 °C. Due to the porosity of the silica gel mixture, the medium and metal can travel through the sol-gel matrix and reach the algal/cyanobacterial cells trapped within the matrix. As shown in Figure 8, the cells remained viable, giving a noticeable fluorescence value each week throughout the 8-week period. Nevertheless, in microalgal cultures *Mesotaenium* sp. 2 and *Mesotaenium* sp. 3 there was no significant difference between the fluorescence values in the first 4 weeks, but a significant difference was observed in the 5th week. Therefore, the storage stability in *Mesotaenium* sp. 2 and *Mesotaenium* sp. 3 was reduced by the 5th week, making the optimum storage time of these two biosensor algal cultures 4 weeks. In the cyanobacterial culture *Synechococcus* sp. no significant difference was observed in all 8 weeks of storage time tested. Hence, the optimum storage time of the cyanobacterial culture can be extended to 8 weeks. Therefore, in this study the biosensor can be stored and reused within a period of 4 weeks without losing the viability of the immobilized cells entrapped in the silica gel matrix of the two microalgal cultures, and the cyanobacterial culture can be stored for 8 weeks. The addition of fresh medium (TM2 in this experiment) helps to revive cells before using the biosensor for the testing of environmental samples. In a study carried out by Durrieu *et al.* (2004) on developing an immobilized biosensor using *Chlorella vulgaris* to monitor wastewater pollutants, it was observed that the biosensor can be used for up to 20 days with 90% remaining activity. Also, in a study by Nguyen-Ngoc & Tran-Minh (2007a) in developing a microalgal biosensor, the algal cells in the silica matrix maintained over 95% of their initial activity even after a period of 5 weeks. Thus, it can be concluded that the biosensor in this study can also be kept over a period of 8 weeks without losing its cell activity. This may be attributed to the fact that viable immobilized cells are able to multiply while remaining confined (to a certain extent) within the immobilization structure, and this ability to grow in the immobilized state makes possible the regeneration of immobilized cell cultures following their operation in hostile incubation conditions such as in a low nutrient medium or the presence of toxic compounds (Junter & Jouenne 2004).

CONCLUSIONS

The microalgal-cyanobacterial array biosensor was successfully developed in the present study. The optimum time of exposure of the biosensor organisms to the contaminant heavy metals was found to be 10 minutes. The three biosensor organisms showed higher survival at high pH values and lower survival at low pH values due to increase in metal toxicity at low pH, which can be attributed to a predominance of free metal ions at acidic pH. When a metal mixture was used instead of a single metal ion on selected microalgae and cyanobacteria, an antagonistic effect between metal ions was observed, reducing the toxic effect of the metal mixture on microorganisms. The immobilized microorganisms in the biosensor maintained their viability throughout 8 weeks (2 months) of storage at 4 °C, making the biosensor stable and reusable even after 8 weeks, after revival of immobilized cells. The optimized biosensor can be used in environmental field monitoring for water samples from industry effluents discharged to inland water bodies to detect the bioavailable fraction of heavy metals. However, the biosensor needs to be characterized and validated for other metals and organics with more environmental samples to obtain more meaningful risk assessment of water bodies and effluents.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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