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Research Article

Whole Cell Biosensor Using *Anabaena torulosa* with Optical Transduction for Environmental Toxicity Evaluation

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A whole cell-based biosensor using *Anabaena torulosa* for the detection of heavy metals (Cu, Pb, and Cd), 2,4-dichlorophenoxyacetate (2,4-D), and chlorpyrifos was constructed. The cyanobacteria were entrapped on a cellulose membrane through filtration. Then, the membrane was dried and fixed into a cylindrical well, which was designed to be attached to an optical probe. The probe was connected to fluorescence spectrometer with optical fibre. The presence of the toxicants was indicated by the change of fluorescence emission, before and after the exposure. The linear detection ranges for Cu, Pb, and Cd were 2.5–10.0 μg/L, 0.5–5.0 μg/L, and 0.5–10.0 μg/L, respectively, while 2,4-D and chlorpyrifos shared similar linear ranges of 0.05–0.75 μg/L. The biosensor showed good sensitivity with the lowest limits of detection (LLD) for Cu, Pb, Cd, 2,4-D and chlorpyrifos determined at 1.195 μg/L, 0.100 μg/L, 0.027 μg/L, 0.025 μg/L, and 0.025 μg/L, respectively. The overall reproducibility of the biosensor ($n = 3$) was $<\pm 6.35\%$. The biosensor had been tested with different combinations of toxicants, with the results showing predominantly antagonistic responses. The results confirmed that the biosensor constructed in this report is suitable to be used in quantitative and qualitative detections of heavy metals and pesticides.

1. Introduction

Industrial and agricultural activities release tonnes of heavy metals and pesticides into the environment. With all these toxicants widespread and threatening the safety of environment, the development of sensitive and fast responding detection devices, which can respond to these toxicants, is pressingly needed.

Conventional analytical equipment for heavy metals and pesticides detection, such as high-performance chromatography and atomic absorption spectrometer is not designed to distinguish whether the toxicants are bioavailable or non-bioavailable to biological systems [1]. In contrast, biosensors are capable of showing the real impact of environment toxicants on living organisms [2, 3].

Whole cell biosensors can be used to detect wide range of changes in the environment and are suitable to be used for the detection of toxicants in the sample from unpredictable resources [4]. Few of the cells' responses that have been utilized in whole cell biosensors are the oxygen release [5], fluorescence emission [6], and enzyme production [7]. The high sensitivity of certain cells to the environmental toxicants such as heavy metals and pesticides has made them good candidates in biosensors [8–11]. By far, some whole cell biosensors have been successfully constructed using bacteria, cyanobacteria, algae, yeast, and strand-off tissue [3, 12–18].

Algae and cyanobacteria are widely available in the environment. These photosynthetic organisms are good candidates to be used as biological components in fluorescence-transduced biosensors [3, 10, 15]. These organisms contain chlorophylls, the pigments which can absorb the energy from light. After absorbing the energy from light, a small portion of the energy absorbed is released as fluorescence emission [19]. According to Campbell et al. [20], the fluorescence yield is depending on the structure of the photosynthetic apparatus, the proximity of the antennae to the reaction centers, and the

concentration and composition of photosynthetic pigments. When the organisms are exposed to toxicants, major photosynthetic electron transport pathways are inhibited. Thus, the fluorescence emission will increase as a way to diffuse the energy which has been absorbed.

Changes of fluorescence have been utilized as detection parameter in biosensor designs [3, 15–17], while cyanobacteria *Anabaena* spp. have been reported to be a potential candidate for biosensor [11, 21]. However, the fluorescence response of *A. torulosa* to heavy metals and pesticides, with simple entrapment on cellulose membrane, has not been reported yet. In this paper, we report the construction of a fluorometric whole cell-based biosensor using *A. torulosa*, which is sensitive to Cu, Cd, Pb, 2-4-D, and chlorpyrifos.

2. Methodology

2.1. A. torulosa Culture. *A. torulosa* was cultured in Bold's basal medium [8, 22, 23]. The culture was maintained in growth chamber (Protech, Malaysia) at 18.5°C with 1000 lux white fluorescent illumination. Light and dark periods were maintained at 16 and 8 hours, respectively. Aeration was carried out daily by manual shaking to prevent the clumping of cells.

2.2. Immobilization of Cyanobacteria and the Construction of Biosensor. Day-7 *A. torulosa* was immobilized onto 3 mm cellulose membrane by filtration. The membrane was air-dried for 24 hours at 18.5°C and punched into small disc with the diameter = 0.6 cm. The number of cells was determined by optical density at 700 nm [11, 24], which initially had been calibrated using microscope (Olympus, USA) and haemocytometer (Weber, UK). The highest fluorescence intensity was produced by the disc which contained 1.1×10^6 cells. Then, the disc with immobilized *A. torulosa* was attached into a round cylindrical well with the diameter ≈ 0.8 cm. The well with the immobilized cells was fixed to an optical probe, which was connected to fluorescence spectrometer (Perkin Elmer, German) with optical fibre. The well was designed for single use because the inhibition of *A. torulosa* by various toxicants was not reversible. This is the usual characteristics of many biosensors based on whole-cell. Figure 1 illustrates the design of biosensor.

2.3. Single Toxicant Detection. The biosensor was operating in room temperature with ambient lighting at pH 7. The fluorescence emission and excitation wavelengths were set at 526 nm and 648 nm. The immobilized *A. torulosa* was activated by adding 10 μL of distilled water to the well. After the activation of cells, the fluorescence intensity before and 30 minutes after the cyanobacteria was exposed to 20 μL of toxicants was measured. The percentage increment of fluorescence was calculated using (1) as shown below:

$$\begin{aligned}\text{Change in fluorescence (\%)} &= \text{\% Fluorescence before the exposure} \\ &\quad - \text{\% Fluorescence after the exposure.}\end{aligned} \tag{1}$$

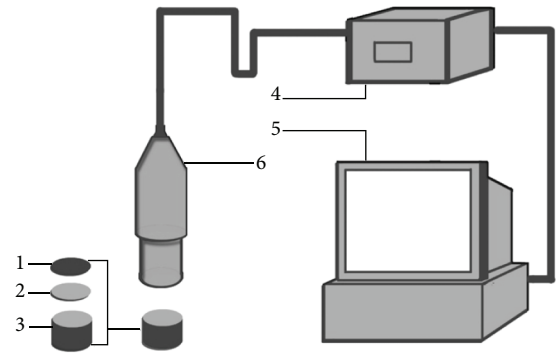

Figure 1: *A. torulosa* (1) is immobilized on cellulose membrane (2), which later has been attached to a well with diameter ≈ 0.8 cm (3). Then, the well is fixed to an optical probe (6) that has been connected to fluorescence spectrometer (4) and computer (5).

2.4. Response of Biosensor to Macronutrients. The biosensor was tested with Ca and Na, respectively. The tests on macronutrients were conducted to compare the responses of the biosensor to the macronutrients and toxicants.

2.5. Combined Toxicants Detection. The biosensor was tested with combined toxicants. Heavy metals (Cu, Pb, and Cd) and pesticides (2,4-D and chlorpyrifos) were mixed in 1 : 1 ratio (v/v) in different combinations. For example, the combination of Cu and Pb was produced by mixing a volume of Cu (with x μg/L) with a same volume of Pb (with x μg/L). Other combinations tested were Cu + Cd, Pb + Cd, Cu + Pb + Cd, Cu + Pb + Cd + chlorpyrifos, and Cu + Pb + Cd + chlorpyrifos + 2,4-D. The effect of combined toxicity was calculated by toxicity unit (TU) [25–27].

3. Results and Discussion

3.1. Biosensor Optimization. The excitation and emission wavelengths of the biosensor were determined experimentally at 526 nm and 648 nm, respectively. The emission wavelength was in agreement with the results obtained by Frense et al. [28] on green algae *Scenesdemus subspicatus* and Kobbia et al. [21] on cyanobacterium *Anabaena variabilis*. The excitation wavelength, however, was different, due to the difference in the species and environmental factors. The test on plain disc (without cyanobacteria) with excitation wavelength of 526 nm showed no fluorescence emission at 648 nm. The result confirmed that the fluorescence emission was produced by *A. torulosa*.

The maximum fluorescence intensity of *A. torulosa* was yielded with 1.1×10^6 cells/disc (Figure 2). At lower density, the increase of cells generally intensified the fluorescence emission. However, the fluorescence intensity started to decrease when the number of cell exceeded 1.1×10^6 cells per disc. The decrease might be a result of the fluorescence emitted which was absorbed by neighbour cells [3]. Based on the result on 1.1×10^6 cells per disc, the optimum activation time for the cyanobacteria was 30 minutes. The best exposure time for the biosensor to the toxicants was 30 minutes (Figure 3).

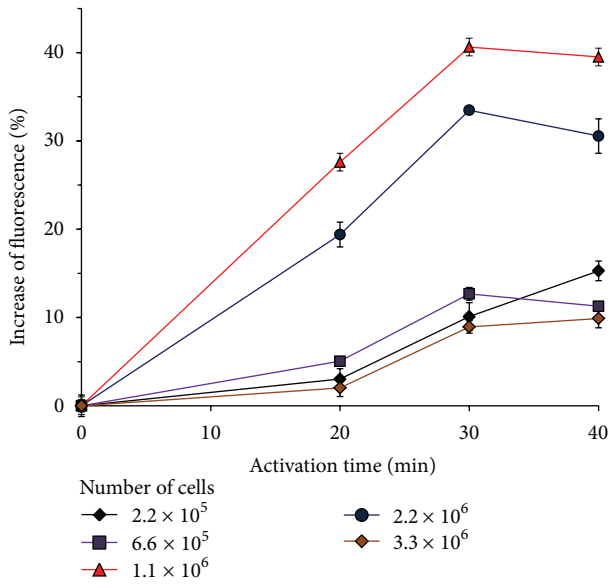

FIGURE 2: The fluorescence response from different amounts of immobilized *A. torulosa* in the cell's activation.

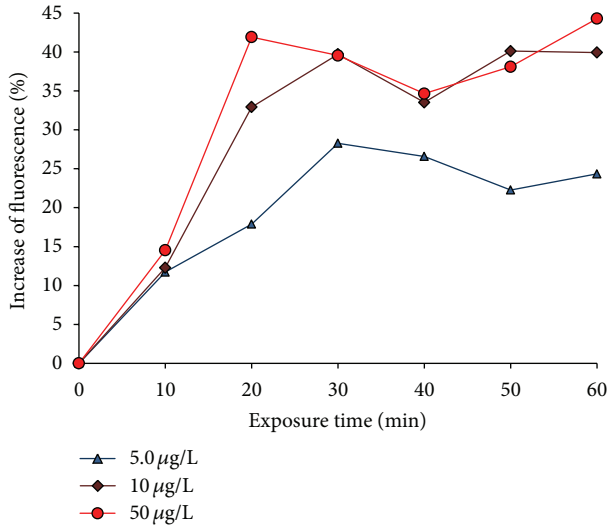

FIGURE 3: Fluorescence response of *A. torulosa* after being exposed to three different concentrations of Cu for 60 minutes.

The durations of activation and exposure for the cells have to be optimized to produce maximum fluorescence emission. Biosensors designed using *Chlorella vulgaris* by Védrine et al. [3] and Chouteau et al. [7] required the activation time of 14 hours and 30 minutes, respectively. The algae biosensor developed by Frense et al. [28] and the bacterium biosensor developed by Philp et al. [12] required exposure time of 5 minutes, while cyanobacteria biosensor developed by Shao et al. [15] recorded exposure time of 24 hours. The exposure times for biosensors are different due to the variation in the design.

3.2. Single Toxicant Detection. The fluorescence response profiles for single toxicant test are shown in Figure 4. The response from the blank is illustrated together with Cd profile. All response profiles shared a close similarity, starting with the increase of fluorescence proportionate to the increase of toxicants until a maximum point, where further increase of toxicants no longer results in higher fluorescence emission. However, for Ca test, fluorescence emission decreases with the increase of Ca (Figure 4). The function of Ca in channeling the energy to phosphorylation pathway in *A. torulosa* might be the factor that leads to the decrease in fluorescence emission. With more energy channeled through phosphorylation, less energy will be released through fluorescence emission [33].

Linear equation, linear detection range, slope, value of r^2, and the LLD for the toxicants tested are shown in Table 1. The value of the slope of linear detection range showed the sensitivity of the biosensor increases with Cu < Cd < Pb < chlorpyrifos < 2,4-D. The biosensor showed lowest sensitivity to Cu as the heavy metal is an essential trace element for plants in small amount to produce metalloproteins. Metalloproteins are important in electron transport or redox reactions [34]. However, high concentration of Cu might effects cell metabolism by interfering the quinone acceptor (QB) in photosystem II [35]. The biosensor showed highest sensitivity to 2,4-D as the compound is highly specific in uncoupling oxidative sites and photosynthetic phosphorylation. The pesticide inhibits the electron transport, thus cut off the energy transduction in PS II [36, 37]. The biosensor showed a good response to chlorpyrifos as well, which was consistent with the result reported by Ma et al. [38].

The LLDs for Cu, Pb, Cd, 2,4-D, and chlorpyrifos were 1.195 μg/L, 0.100 μg/L, 0.027 μg/L, 0.025 μg/L and 0.025 μg/L, respectively. As the World Health Organization (WHO) [39] guidelines for drinking water quality have set the limits for Cu, Pb, Cd, and 2,4-D at 2000.0 μg/L, 10.0 μg/L, 3.0 μg/L, and 30.0 μg/L, respectively, the biosensor constructed could operate within the safety standard required by WHO. Table 2 shows the LLD of the biosensor constructed in this work and several other whole cell-based biosensors. The biosensor had good reproducibility with the average standard deviation (n = 3) <±6.35%. The reproducibility of the biosensor constructed was close to the reproducibility of the whole cell biosensors designed by Védrine et al. [3] and Chay et al. [11].

The biosensor constructed in this work showed competitive results compared to the other biosensors using different biological components as reporting group, such as the optical enzyme biosensors developed by Tsai et al. [40] and Zhylyak et al. [41] for Cu, Cd, and Pb detection.

When the biosensor tested Ca, a decrease in fluorescence was observed (Figure 4). The biosensor responded to Na in a similar trend. Faller et al. [42] reported that the heavy metals compete with Ca on the same binding site and give a totally opposite effect to photosynthesis. Therefore, the presence of macronutrients increases the photosynthesis rate and reduces the intensity of fluorescence.

3.3. Combined Toxicants Detection. The combined toxicity effects on biosensor can be synergistic, additive, or

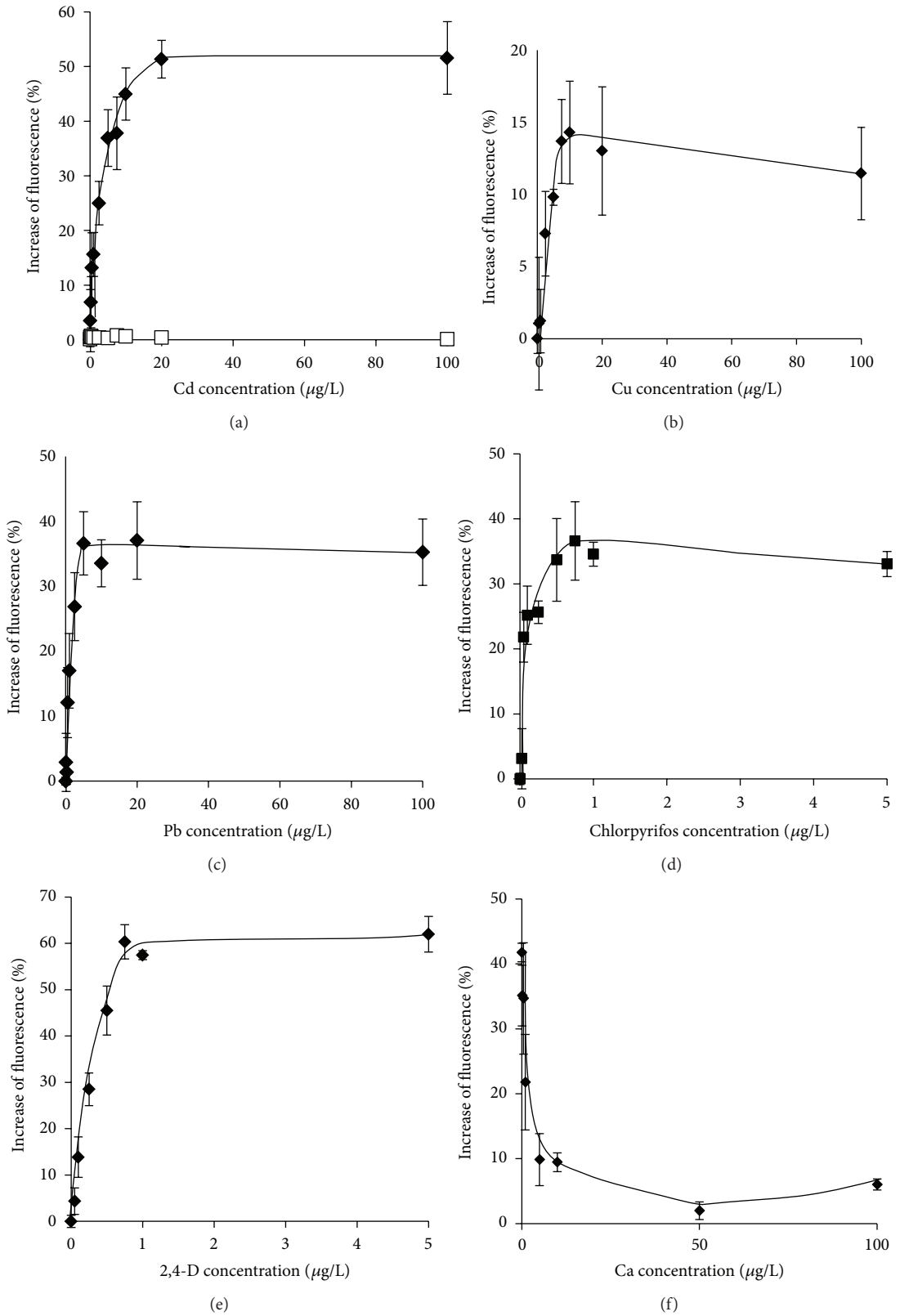

Figure 4: The response profiles for Cd, Pb, Cu, 2,4-D, and chlorpyrifos. The square boxes in Cd profile represent the fluorescence response of blank for the tests.

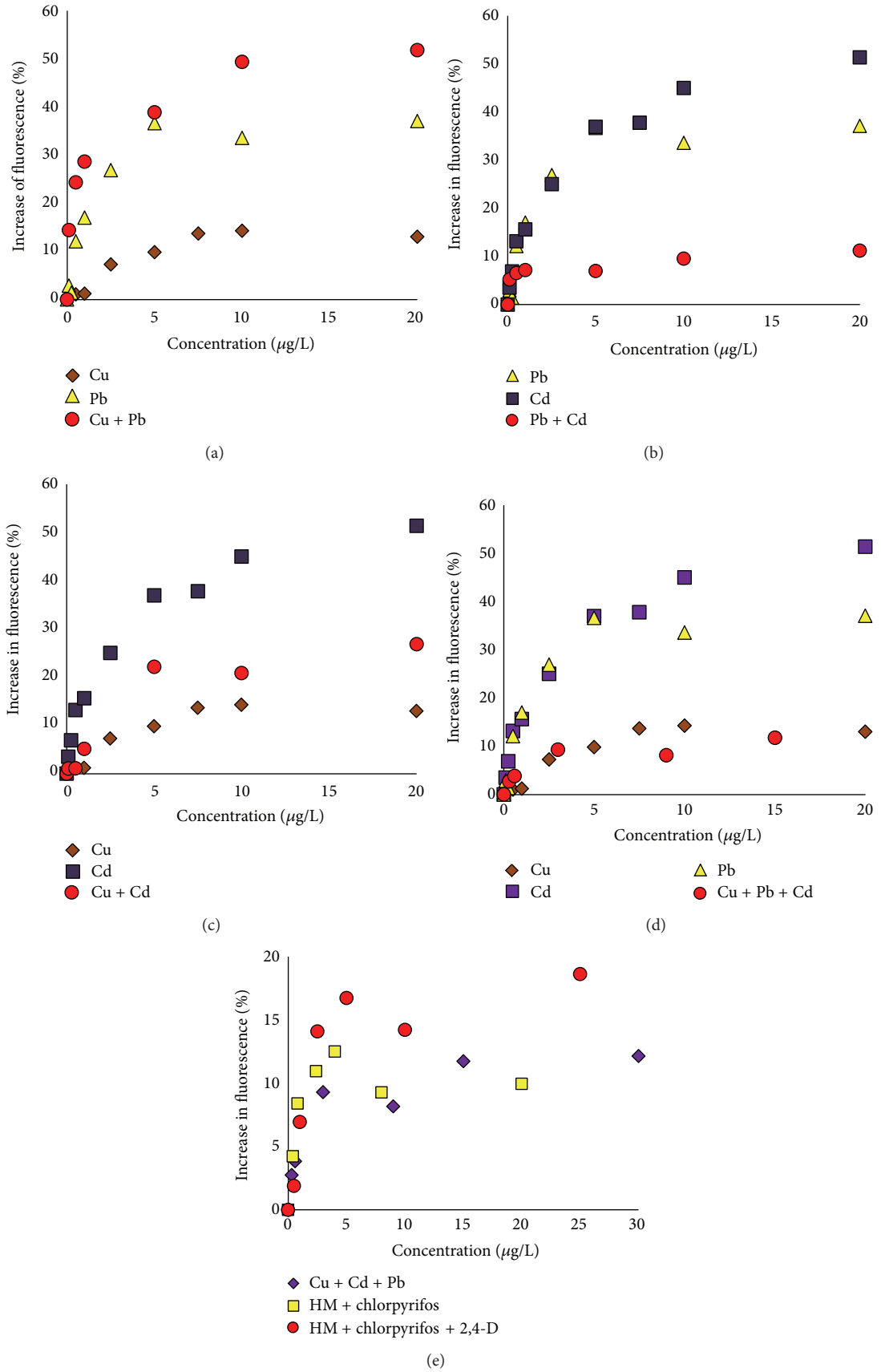

Figure 5: The comparison between the response profiles of combination toxicants and the single toxicant.

Table 1: Linear equation, linear detection range, slope, value of r^2, and the LLD for the detection of toxicants.

Toxicants	Linear equation	Linear detection range (μg/L)	Slope	r^2	LLD (μg/L)
Cu	$y = 0.995x + 5.040$	2.50–10.00	0.995	0.940	1.195
Cd	$y = 3.295x + 14.35$	0.50–10.00	3.295	0.970	0.100
Pb	$y = 5.31x + 11.19$	0.50–5.00	5.310	0.926	0.027
2,4-D	$y = 76.93x + 5.128$	0.05–0.750	6.930	0.976	0.025
Chlorpyrifos	$y = 20.88x + 21.68$	0.05–0.750	0.882	0.952	0.025

Table 2: Lowest limits of detection of several whole cell-based biosensors.

Organism	Transducer	Lowest limit of detection μg/L					Reference
		Cu	Cd	Pb	2,4-D	Chlorpyrifos	
C. vulgaris	Optical				1		Frense et al. [28]
Spirulina subsalsa	Amperometric	100					Campanella et al. [29]
Recombinant *Escherichia coli*	Optical				1200		Strachan et al. [16]
C. vulgaris	Optical		100	10			Durrieu and Tran-Minh [30]
C. vulgaris	Amperometric		10				Chouteau et al. [7]
A. torulosa	Amperometric	300					Chay et al. [11]
E. coli	Amperometric	1000					Wang et al. [31]
Recombinant *Tetrahymena thermophila*	Optical	95.00	0.56	10.36			Amaro et al. [32]
A. torulosa	Optical	1.195	0.100	0.027	0.025	0.025	This work

antagonistic [43]. The tests of biosensor on combined toxicants (Cu + Pb, Cu + Cd, Pb + Cd, Cu + Pb + Cd, Cu + Pb + Cd + chlorpyrifos, and Cu + Pb + Cd + chlorpyrifos + 2,4-D) showed predominantly antagonistic effects.

Figure 5 depicts the response of the biosensor to the individual toxicants, together with combined toxicants. The toxicity effects were calculated using the value of EC_{50} (combined toxicants)/ΣTU (individual toxicant). The toxicity effects increased by Cu + Pb + Cd + chlorpyrifos (0.054) < Cu + Pb + Cd + chlorpyrifos + 2,4-D (0.175) < Cu + Pb (0.332) < Pb + Cd (0.400) Cu + Pb + Cd (0.422) < Cu + Cd (0.6117). The presence of Cd in the combination of heavy metals produced higher response, with Cu + Cd recording the highest increase in fluorescence, followed by Cu + Pb + Cd and Pb + Cd. The combination of Cu + Pb produced the least fluorescence compared to other combinations of heavy metals. The results due to the copresence of Cu and Pb had diminished the inhibition of photosystems in cyanobacteria, while copresence of Cu and Cd had increased the inhibition. The results were in agreement with the work reported by Chaperon and Sauvé [44].

The antagonistic results from the combinations of heavy metals with organic pesticides were expected. The copresence of heavy metals and organic pesticides led to the formation of organic substance-heavy metal complex [27]. The complex was less toxic to the cyanobacteria and thus produced lower fluorescence emission.

3.4. Stability of the Biosensor. Wells with immobilized *A. torulosa* were stored to be tested for the performance from day 1 to day 25 with a 5-day interval. The wells were tested with single exposure to Cu 5.0 μg/L. The performance of the biosensor diminished significantly after the first 5 days of storage, while it reached a more stable state after 20 days of storage. The decrease in performance could be fitted into the equation $y = -2.3306 \ln(x) + 13.078$, with high correlation between the duration of storage to the performance of biosensor ($r^2 = 0.9863$). The decrease of performance was expected as the biological component degraded as the storage time increased. The same trend was observed on other whole cell-based biosensors, as reported by Chouteau et al. [7], Reshetilov et al. [45], and Akyilmaz et al. [46].

4. Conclusion

A whole cell biosensor is constructed using cyanobacteria *A. torulosa* using fluorescence spectrometer as the transducer. The biosensor operated under optimized condition with cells from day-7 culture, 1.1×10^6 cells per disc, and the activation and the exposure times were both set at 30 minutes, respectively. The biosensor showed high sensitivity towards heavy metals and pesticides with the LLD fall within the range of 0.025–1.195 μg/L. The linear detection ranges showed that the biosensor is capable of being used for qualitative and quantitative detections. Although the biosensor responded antagonistically towards the combination of toxicants, the

results showed that the qualitative detection for the mixture of toxicants is possible.

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