

## The Fluorometric Response of Cyanobacteria To Short Exposure of Heavy Metal

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### ABSTRACT

This study is focused on the response of cyanobacteria *Anabaena torulosa* to a short exposure to copper. Measurement was based on the differential of the fluorescence signal of the organism before and after the exposure to copper, with excitation and emission set at 526 nm and 648 nm respectively. To optimize the yield of fluorescence signal, the day-7 culture of cell suspension was used with the density of  $OD_{700nm} = 0.45$  A, with short exposure time of 8 minutes to Cu. Tests on different concentration of Cu showed the cyanobacteria fluorescence yields formed a linear range from 1 – 10  $\mu\text{g/L}$ . The cyanobacteria could be a good candidate for biosensor for producing signals under a short exposure to low concentration of Cu.

**Key words:** Cyanobacteria, fluorometric response, heavy metal, short exposure time.

### Introduction

As a tool designed for rapid assessment of the presence of toxics, the response time in developing a biosensor is always a challenge. A few cyanobacteria have been reported as a potential candidate to be used in biosensor [3,12,25,33]. However, the response of cyanobacteria to short period of exposure to heavy metals is yet to study. The objective of this study is to determine the fluorometric response of cyanobacteria in suspension to Cu in a short period of time. The study is very important to identify the possibility of the cyanobacteria *Anabaena torulosa* to be used in biosensor.

Cyanobacteria or blue-green algae, are prokaryotes with high diversity and adapted to many habitats, which has existed for millions of years [32,15]. Cyanobacteria are chlorophyll containing photosynthetic organisms with the ability to produce oxygen [2]. The chlorophylls in cyanobacteria serve as the receivers to the photons from the sun [30]. Most of the potential energy received is diffused through the conversion to heat, while some of the potential energy cascade down through a electron transport chain, which ends up with the production of NADPH. A small amount of the potential energy is channeled through the emission of fluorescence.

The chlorophyll fluorescence occurs when an excited electron returns to its resting state through photon emission [15]. According to Planck equation, the photon emitted through fluorescence with lower energy has longer wavelength compared to the

wavelength of the photon absorbed. The *in vivo* studies conducted by Evan and Brown [7] and Krause and Weis [13] showed the chlorophyll fluorescence is contributed significantly by PSII, where the  $P680^+$  is reduced to  $P680$ . In biological system, chlorophyll fluorescence is low.

The presence of heavy metals are reported to inhibit the photosynthesis by the binding to the oxidation sites and the reduction of PSII [1,4,14]. Yatsenko [34] stated that the heavy metals stop photosynthesis by inhibiting the -SH containing proteins. *In vitro* experiment carried out by Kimimura and Katoh [11] confirmed that the heavy metals are able to inhibit plastocyanin, which appears to be an important electron transporter in photosystems. As the presence of heavy metals inhibits the electron transport chain in photosynthesis, hence increases the fluorescence emission as an alternative channel to drain the energy collected from the sun.

In this study, the Cu was selected as the representative of heavy metals. The heavy metal is less toxic to the plants, as Cu is required in small quantity for the plants in the synthesis of metalloproteins [5]. The response of the *A. torulosa* to Cu could serve as a reference to other heavy metals, which are more toxic to cyanobacteria.

### Material and Methods

*The Culture and the Determination of the Cell Growth:*

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The cyanobacteria *A. torulosa* was cultured in Bold Basic Medium at 18.5°C, which was modified from Bristol Medium to provide all the essential nutrients for the growth of the cyanobacteria [10,25,27]. Light and darkness were maintained at 16 hours and 8 hours intervals respectively. Manual aeration was carried out twice daily to prevent the clumping of the colonies. The cell growth was determined by cell count with haemocytometer. The count was made every two days for 16 consecutive days. The cyanobacteria from day-7 culture, which was in its exponential growth phase was selected to be used in this study. The number of the cyanobacteria were later been correlated to the optical density (OD) at the wavelength 700 nm using spectrophotometer (Perkin Elmer, Germany).

#### *The Optimization of the Number of Cell for Spectrofluorometer:*

Using spectrofluorometer (Perkin Elmer, Germany), the intensity of fluorescence emission for the culture containing *A. torulosa* was determined at 648 nm, with the excitation wavelength set at 526 nm. In order to identify the excitation wavelength, an excitation wavelength scan was conducted with the emission wavelength set at 600 – 700 nm, which is the range of the chlorophyll fluorescence emission [8,12,29,35]. From the scanning result, the excitation wavelength at 526 nm was determined. The excitation wavelength at 526 nm was then used to determine the exact wavelength of fluorescence emission, which was at 648 nm.

For the optimization, a volume of 4 mL of the culture medium containing *A. torulosa* in suspension with the OD<sub>700nm</sub> = 0.10, 0.30, 0.40, 0.45, 0.50, 0.75, 1.00, 1.50, and 2.00 A were prepared. The intensity of the fluorescence emission of the cyanobacteria with different OD at 700 nm were determined respectively using spectrofluorometer, which the cyanobacteria at OD<sub>700nm</sub> = 0.45 A (equivalent to  $4.72 \times 10^6$  sel/ mL) yielded the highest intensity of fluorescence emission, thus was selected as the optimized condition for this study.

#### *The Optimization of the Exposure Time:*

A volume of 4 mL of *A. torulosa* in suspension was transferred into a four-sided clear quartz cuvette. A small volume of Cu solution was introduced to the cell in the cuvette, making the final concentration of 1 µg/L, 10 µg/L, and 100 µg/L of Cu. The intensity of the fluorescence emission for the cyanobacteria was measured before the exposure to Cu. The measurements were made after 5, 10, and 15 minutes of the exposure respectively. In this study, the exposure time of *A. torulosa* to Cu was set at 8 minutes, which is in between 5 – 10 minutes.

#### *The Measurement of the Fluorometric Response to Heavy Metal:*

The culture of *A. torulosa* at the final concentrations of 1, 2, 4, 6, 8, 10, 12, and 14 µg/L of Cu were prepared by adding a small volume of Cu solution. The intensity of the fluorescence emission was measured before the exposure and after 8 minutes of exposure. The change of the intensity of the fluorescence emission was calculated by  $F - F_0$ , where F represents the fluorescence after the exposure and  $F_0$  represents the fluorescence before the exposure. The percentage of the change of the intensity of the fluorescence emission was calculated by  $(F - F_0) \times 100\% / F$ .

### **Results and Discussion**

The growth of *A. torulosa* can be categorized into three different phases, namely the lag phase, the exponential phase and the stationary phase. The growth of the cyanobacteria was slow for the first four days after the subculture (Figure 1), followed by the rapid exponential growth from day-4 to day-8, and entered the stationary phase from day-8 to day-16, where day-0 indicates the day where subculture was carried out. According to Tortora *et al.*, [26] microbes are most sensitive to the environmental changes at their exponential phase. The usage cyanobacteria at the exponential growth phase for toxicity tests had been reported by many [25,23,33,19]. Therefore, the cyanobacteria from the day-7 culture was selected to be used in this study.

The number of cell can be determined by the reading of OD at 700 nm, which is the absorption wavelength for chlorophyll *a*. Figure 2 delineates the correlation between the number of *A. torulosa* is shown in. The positive correlation shows the increment of chlorophyll captured by OD at 700 nm is brought by the growth of the cyanobacteria. Despite lower in sensitivity, estimating the number of cell using the reading of OD is more convenient than using haemocytometer [26]. The reading of OD had been used to determine the number of fluorescence bacteria [9,18], green algae [29] and cyanobacteria [25]. The value of  $r^2 = 0.9945$  shows the increment of the OD at 700 nm is linearly correlated with the increment of the cyanobacteria, with the linear equation of  $1 \times 10^7 x + 217558$ .

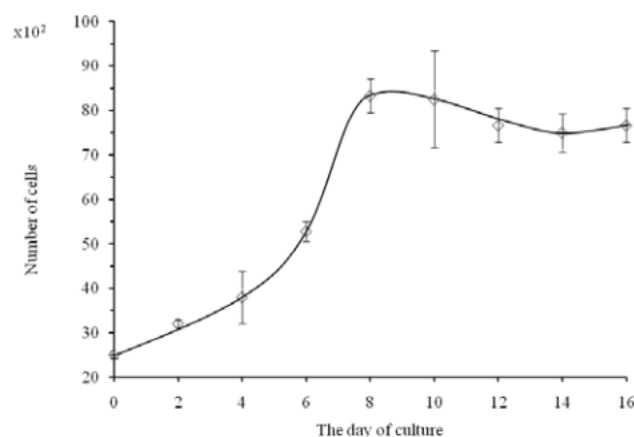
The correlation between the number of *A. torulosa* (in OD<sub>700 nm</sub>) is portrayed in Figure 3. From OD<sub>700nm</sub> = 0.10 – 2.0 A. The intensity of the fluorescence increased dramatically, which mostly contributed by the increment of the amount of chlorophyll *a* in the cyanobacteria. However, when the increment of the cyanobacteria exceeded 0.45 A, non-photochemistry quenching, where the emitted fluorescence was reabsorbed by the receptors nearby lowered the fluorescence intensity [20,29]. Consequently, the intensity of the fluorescence decreased from OD<sub>700 nm</sub> = 0.45 – 2.0 A. Result shown the cyanobacteria culture at OD<sub>700 nm</sub> = 0.45 A, which equivalent to  $4.72 \times 10^6$  cells/mL yielded maximum intensity of fluorescence, thus been set as the optimized OD at 700 nm for the experiment.

The culture at day-7 containing *A. torulosa* in suspension with  $OD_{700\text{ nm}} = 0.45$  A was exposed to three different concentration of Cu for 15 minutes, with the results depicted in Figure 4. The concentration of Cu affected the yield of fluorescence intensity, as the fluorescence intensity at 10  $\mu\text{g/L}$  of Cu was higher than 1  $\mu\text{g/L}$  of Cu. However the addition of Cu to 100  $\mu\text{g/L}$ , a decrease in fluorescence intensity was observed. The low fluorescence intensity yielded at higher concentration of Cu (10  $\mu\text{g/L}$ ) might caused by the photo-chemical reaction of cyanobacteria in the presence of high concentration of Cu [22]. Tauber *ET AL.*, [24] reported the decrease of fluorescence was obvious for fluorometric microorganisms in high concentration of certain toxics. For all three concentrations of Cu, the maximum yield of fluorescence intensity were reached within 5 to 10 minutes of exposure. Therefore, the exposure time for further experiments was set at 8 minutes.

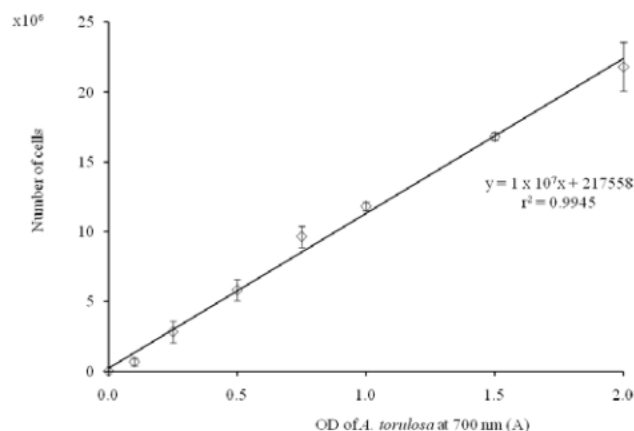
The cyanobacteria in suspension then been exposed to different concentrations of Cu ranged from 1 – 14  $\mu\text{g/L}$ , with a steady climb in the intensity of fluorescence from 1 – 10  $\mu\text{g/L}$  with saturation showed from 10 – 14  $\mu\text{g/L}$  (Figure 5). A linear

fluorometric response was identified at the range of 2 – 10  $\mu\text{g/L}$  (Figure 6). Within the linear response range, the fluorescence intensity raised from 2.39 % to 5.09 % compared to the control (without Cu). However, using the detection limit calculation demonstrated by Miller and Miller [16], the detection limit of the cyanobacteria was tuned to 2.21  $\mu\text{g/L}$ . Therefore, the linear response range of the cyanobacteria to Cu was 2.21 – 10.00  $\mu\text{g/L}$ , with the percentage of increment of fluorescence intensity correlated to the increment of Cu with the equation  $y = 0.446x + 1.1816$ . The value of  $r^2 = 0.9733$  showed a high correlation between the increment of fluorescence intensity to the change of Cu concentrations.

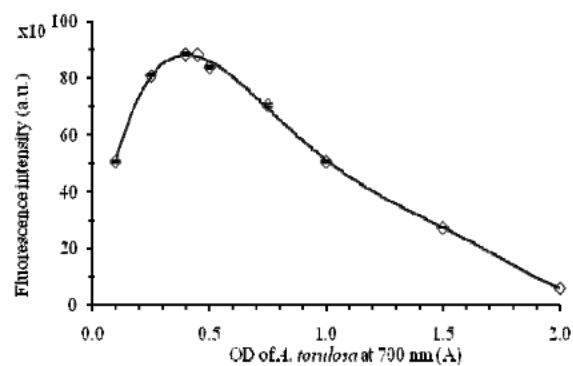
The response of *A. torulosa* in suspension to short exposure to Cu was confirmed, with the optimized condition using day-7 culture with  $OD_{700\text{ nm}} = 0.45$  A, and the exposure time of 8 minutes. The response was in the form of the increment of fluorescence intensity, caused by the inhibition of Cu towards the process of photosynthetic electron transport chain [21,28]. The inhibition increases the intensity of the fluorescence emission at 648 nm.



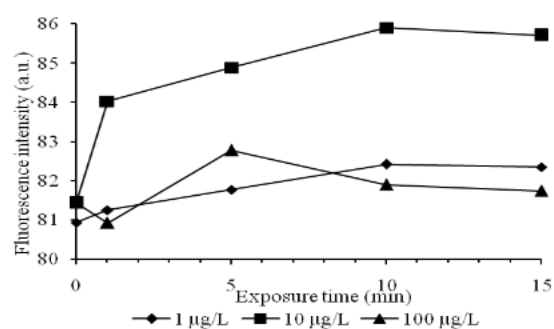
**Fig. 1:** The growth curve of *A. torulosa* from the subculture at day-0 to day-16.



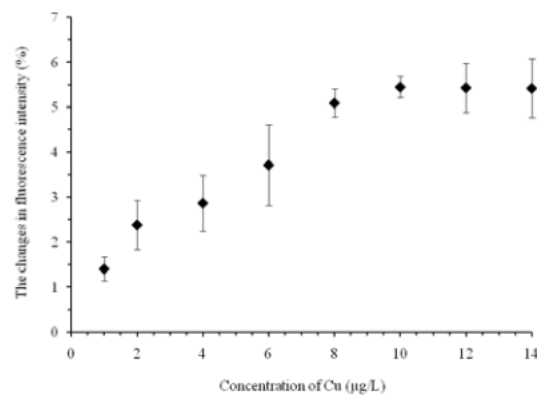
**Fig. 2:** The correlation between OD with the number of cells.



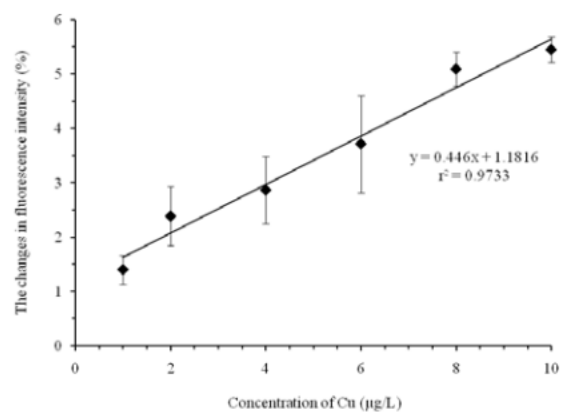
**Fig. 3:** OD at 700 nm and the fluorescence intensity.



**Fig. 4:** Fluorescence changes of *A. torulosa* under 15 minutes exposure to three different concentration of Cu.



**Fig. 5:** The fluorometric response of *A. torulosa* to Cu from 1 µg/L – 14 µg/L.



**Fig. 6:** The linear fluorometric response range of *A. torulosa* to Cu.

The response of *A. torulosa* to Cu showed that the cyanobacteria were able to detect the presence of Cu from 2.21 – 100.00 µg/L qualitatively. The cyanobacteria were capable to detect the presence of Cu quantitatively within the range of 2.21 – 10.00 µg/L. In addition to that, *A. torulosa* responded to the Cu at the concentration as low as 2.21 µg/L met the requirement of Class II standard for drinking water set by National Water Quality Standards for Malaysia [6]. So, the fast fluorometric response of *A. torulosa* to Cu was a good indication, showing that *A. torulosa* is a good candidate for the development of biosensor. Compared to the work done by Tay *et al.*, [25] and Wong *et al.*, [33] on the amperometric biosensor using the same cyanobacteria, the optical approach utilized in this study yielded the stable reading almost half of the time that the amperometric approach did.

#### Conclusion:

The result of this study confirmed that the cyanobacteria *A. torulosa* responded to the short period of exposure to Cu, by increasing the yield of the fluorescence emission. The cyanobacteria was able to give detectable fluorescence signal to as low as 2.21 µg/L of Cu as well. The sensitivity of this cyanobacteria to Cu might be an indication for even higher sensitivity on other heavy metals which demonstrate higher toxicity effect on the cyanobacteria. The high sensitivity of the cyanobacteria to Cu make it a promising candidate to be use as heavy metals biosensor for drinking water.

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