

**THE DEVELOPMENT OF A BIOSENSOR FOR  
THE DETECTION OF PS II HERBICIDES  
USING GREEN MICROALGAE**

PERPUSTAKAAN  
UNIVERSITI MALAYSIA SABAH



**MAIZATUL SURIZA MOHAMED**

**UMS**  
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**SEKOLAH SAINS DAN TEKNOLOGI  
UNIVERSITI MALAYSIA SABAH  
2006**

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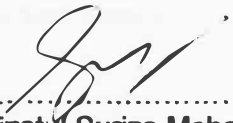
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## Abstrak

### **PEMBANGUNAN BIOSENSOR UNTUK MENGESAN HERBISID FOTOSISTEM II DENGAN MENGGUNAKAN ALGA HIJAU**

Biosensor PS II telah dibangun menggunakan sel alga hijau dari filum Chlorophyta. Biosensor ini adalah berdasarkan kemampuan dan kecenderungan sesetengah residu herbisid (herbisid fotosistem II) merencat tapak pelekatan penerima/penderma elektron primer fotosistem II, plastokuinon pada protein heterodimer D1. Perencatan ini menyakibatkan kenaikan "fluorescence" klorofil. "Fluorescence" klorofil yang dijanakan dari perencatan ini, kemudiannya dikorelasikan dengan kepekatan herbisid yang digunakan untuk membina satu keluk kalibrasi piawai yang akan digunakan dalam penentuan kepekatan herbisid. Parameter yang digunakan untuk mewakili nilai "fluorescence" yang diperolehi adalah kadar kenaikan "fluorescence" yang dikira secara matematik daripada keluk kenaikan "fluorescence"-masa yang diperolehi daripada eksperimen. Bacaan fluorescence diambil menggunakan fluorometer TD-700, Turner design, USA. Enam alga air tawar diasingkan dari tasik berdekatan Teluk Likas, Kota Kinabalu, Sabah dan digunakan dalam kajian iaitu *Chlorella* sp., *Pediastrum* sp., *Kirchneriella* sp., *Coelastrum* sp., *Scenedesmus dimorphus*, and *Selenastrum* sp. *Scenedesmus dimorphus*. Daripada kajian yang dijalankan, *Scenedesmus dimorphus* didapati paling sesuai digunakan sebagai biosensor berdasarkan kriteria-kriteria berikut: (1) kadar pertumbuhan yang tinggi, (2) mudah dikultur dan mengekalkan ketulenan, dan (3) sensitif kepada kepekatan herbisid yang rendah. Kesan umur kultur sel, suhu kultur, pelarut organik, matrik sampel dan masa pra-eraman gelap sampel turut dikaji untuk menyelaraskan biosensor yang dibina. Dengan menggunakan kultur sel *Scenedesmus dimorphus* berusia 14 hari, hubungan linear di antara nilai  $\log_{10}$  fluorescence yield kepada nilai  $\log_{10}$  kepekatan herbisid diperolehi. Daripada keluk hubungan ini, didapati kepekatan terendah yang boleh dikesan menggunakan biosensor *Scenedesmus dimorphus* ini adalah 0.001mM untuk diuron dan 0.01 mM untuk propanil dan bromacil. Biosensor ini boleh digunakan sebagai pengesanan awal racun rumpai jenis PS II dan mempunyai potensi yang baik untuk menjadi kaedah pengesanan racun rumpai yang mudah, murah dan cepat pada masa akan datang.

## Abstract

### THE DEVELOPMENT OF A BIOSENSOR FOR THE DETECTION OF PS II HERBICIDES USING GREEN MICROALGAE

A PS II biosensor was developed using intact green algae of Chlorophyta. The biosensor was based on the ability of some herbicides (PS II herbicides) residue to inhibit the binding niche of primary electron donor/acceptor of Photosystem II, plastoquinone B ( $Q_B$ ) at D1 heterodimer protein, causing an increase of the chlorophyll fluorescence. Herbicide-induced chlorophyll-a fluorescence was correlated with the corresponding herbicide concentration to obtain a standard calibration curve for herbicide detection. The fluorescence yield was expressed as a rate of the fluorescence increase calculated mathematically from fitted curve of herbicide-induced fluorescence data using SigmaPlot and CurveFit Expert software. The fluorescence was recorded using a TD-700 fluorometer from Turner Design, USA. Six freshwater microalgae isolated from a lake near Likas Bay, Kota Kinabalu, Sabah were examined; *Chlorella* sp., *Pediastrum* sp., *Kirchneriella* sp., *Coelastrum* sp., *Scenedesmus dimorphus*, and *Selenastrum* sp. Of all the species examined, *Scenedesmus dimorphus* was found to be the most suitable algae as a biosensor based on the following criteria: (1) highest growth rate, (2) easy to culture and maintain purity, and (3) sensitivity to low concentration of herbicide. Effects of culture age, temperature, solvents, sample matrix and pre-incubation of sample were examined to optimize the biosensor. Cell suspension at 14-days was found to be the best algal age especially when using *Scenedesmus dimorphus*. The correlations between fluorescence yields (expressed as the rate of fluorescence,  $\beta$ ) and concentration ( $\alpha$ ) of diuron, propanil and bromacil were  $\alpha = 10^{\wedge}((\log \beta - 0.536)/1.08)$ ,  $\alpha = 10^{\wedge}((\log \beta - 0.3893)/1.1119)$  and  $\alpha = 10^{\wedge}((\log \beta - 0.1678)/0.7084)$ , respectively. The detection range of diuron, propanil and bromacil were  $1 \times 10^{-3}$  mM – 1mM,  $1 \times 10^{-1}$  mM –  $1 \times 10^1$  mM and  $1 \times 10^{-1}$  mM –  $1 \times 10^2$  mM, respectively. The lowest detectable concentration of the biosensor was approximately  $1 \times 10^{-3}$  mM ( $2.33 \times 10^2$   $\mu$ g/L), for diuron,  $1 \times 10^{-1}$  mM ( $2.18 \times 10^3$   $\mu$ g/L) for propanil and  $1 \times 10^{-1}$  mM ( $2.61 \times 10^3$   $\mu$ g/L) bromacil. The biosensor can be used as an early warning system at present and offers a potential rapid, cheap and fast method for PS II herbicides detection in the future.

## LIST OF ABBREVIATIONS

<b>2,4-D</b>	2,4-dichlorophenoxy acetic acid
<b>ANOVA</b>	Analysis of Variance
<b>DCMU</b>	3-(3,4-dichlorophenyl)-1,1 dimethylurea
<b>DMSO</b>	dimethyl sulfoxide
<b>DNA</b>	deoxyribonucleic acid
<b>ELISA</b>	Enzyme-Linked Immunosorbent Assay
<b>EPTC</b>	S-ethyl dipropylthiocarbamate
<b>GC</b>	Gas Chromatography
<b>GCMS</b>	Gas Chromatography – Mass Spectrometry
<b>HPLC</b>	High Performance Liquid Chromatography
<b>HRAC</b>	Herbicide Resistance Action Committee
<b>LED</b>	light-emitting diode
<b>MCPA</b>	2-methyl-4-chlorophenoxyacetic acid.
<b>MADA</b>	Muda Agricultural Development Authority
<b>NADP</b>	nicotinamide adenine dinucleotide phosphate
<b>NADPH</b>	nicotinamide adenine dinucleotide phosphate, reduced form
<b>P<sub>680</sub></b>	Chlorophyll pigment 680
<b>P<sub>700</sub></b>	Chlorophyll pigment 700
<b>PC</b>	plastocyanin
<b>ppb</b>	part per billion
<b>ppm</b>	part per million
<b>PQ</b>	plastoquinone
<b>PQH<sup>-</sup></b>	quinone anion radical
<b>PQH<sub>2</sub></b>	reduced plastoquinone
<b>PS I</b>	Photosystem I
<b>PS II</b>	Photosystem II
<b>Q</b>	quinone
<b>Q<sub>A</sub></b>	Plastoquinone A
<b>Q<sub>B</sub></b>	Plastoquinone B
<b>rpm</b>	rotation per minutes
<b>SPSS</b>	Statistical Package for the Social Sciences
<b>Std. dev</b>	standard deviation



<b>Std. error</b>	standard error
<b>U.S. EPA</b>	Environmental Protection Agency, United States of America
<b>Y<sub>z</sub></b>	redox-active tyrosine
<b>LHIIC</b>	Light harvesting protein-chlorophyll complex



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## LIST OF SYMBOLS

%	percentage
>	greater than
°	degree
° C	degree Celsius
µg/L	microgram per liter
µg/ml (or µg/mL)	microgram per milliliter
µl (or µL)	microliter
µm	micrometer
µM	micro-molar
Å	Armstrong
$F_{\max}$	maximum fluorescence
$F_0$	constant fluorescence (initial)
fsu	fluorescence unit
$F_v$	variable fluorescence
g	grams
g	generation time
h	hour (s)
k	growth rate constant
kD	kilo Dalton
ml (or mL)	milliliter
mm	millimeter
mM	millimolar
nM	nanomolar
$r^2$	correlation coefficient
v/v	volume per volume



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# CHAPTER 1

## INTRODUCTION

Pesticides are toxic chemicals that are widely used today to help us deal with unwanted plants or animals in order to protect our interest. They include various types of chemicals: organic (mostly synthetic), inorganic and organometalic which can be further divided into sub-categories such as herbicides, insecticides, rodenticides, fungicides, nematocides and algaecides, depending on the organism they can kill. They cover a variety of pest-control strategies, as shown in Table 1.1. Indiscriminate usage of pesticides cause negative effect to human, environment and the whole ecosystem since pesticides accumulation in tissue in high concentrations can cause severe damage (Schmid *et al.* 1990).

A recent survey on pesticides usage and associated incidence of poisoning in the Muda area indicated that herbicides were most frequently used compared to insecticides, fungicides, and rodenticides (Ho, 1998). Most of the herbicides are very toxic and carcinogenic. Sales of pesticides in Malaysia in year 1980 amounted to RM160 million (Lee and Ong, 1983). Among these, herbicides usages were the highest. In 1995, in the Muda Irrigation Scheme, 646 metric ton of herbicides were used to control various weeds in the paddy field (Ho, 1998). Table 1.2 shows the usage of different types of herbicides from 1980 to 1995 in the Muda Irrigation Scheme. Massive usage of herbicides in agriculture has become serious

Table 1.2 Estimated usages of herbicides in the Muda Irrigation Scheme, Malaysia (in metric tons) (modified from Ho, 1998)

Type of herbicide	Mode of action	Year													
		1980	1983	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	
2,4-D IBE	Synthetic auxin (growth regulator)	100	180	250	280	250	250	200	160	150	150	150	140	145	
2,4-D Sodium Salt	Synthetic auxin (growth regulator)	50	20	NA	NA	NA	30	10	8	5	4	4	4	4	
2,4-D Amine	Synthetic auxin (growth regulator)	5	20	150	150	140	130	60	50	40	30	30	30	30	
MCPA	Synthetic auxin (growth regulator)	1	NA	1	1	1	-	-	-	-	-	-	-	-	
Molinate	Inhibition of lipid synthesis - not ACCase inhibition	-	10	110	195	265	380	600	500	550	250	240	70	50	
Molinate & Propanil	Inhibition of lipid synthesis - not ACCase inhibition and inhibition of photosynthesis at photosystem II	-	-	-	-	-	8	10	10	10	5	6	5	5	
Oxadiazon	Inhibition of protoporphyrinogen oxidase (PPO)	-	-	10	10	6	6	6	2	2	-	-	-	-	
Propanil	Inhibition of photosynthesis at photosystem II	-	-	-	2	14	20	22	26	25	20	25	20	15	
Thiobencarb	Inhibition of lipid synthesis (not ACCase inhibition)	-	-	-	-	-	50	50	10	-	-	-	10	5	
Thiobencarb & Propanil	Inhibition of lipid synthesis (not ACCase inhibition) and inhibition of photosynthesis at photosystem II	-	-	-	-	-	-	-	-	-	6	8	30	45	
EPTC	Inhibition of lipid synthesis (not ACCase inhibition)	-	-	-	-	-	-	20	19	20	50	58	40	40	
Pretilachlor	Inhibition of cell division (inhibition of VLCFAs)	-	-	-	-	-	-	5	6	5	5	7	8	8	
Paraquat	Photosystem I electron diversion	10	80	300	320	320	320	330	300	270	250	225	250	240	
Glyphosate	Inhibition of EPSP synthase	-	-	-	-	-	-	-	-	-	20	25	30	40	
Fenoxaprop	Inhibition of acetyl Co A carboxylase (ACCCase)	-	-	-	-	-	-	-	-	7	5	8	6	6	
Sethoxydim	Inhibition of acetyl Co A carboxylase (ACCCase)	-	-	-	-	-	-	-	-	-	8	3	2	-	
Quinchlorac	Synthetic auxin (growth regulator)/ Inhibition of cell wall (cellulose) synthesis	-	-	-	-	-	-	-	-	-	2	2	1	1	
Others	-	NA	NA	1	2	3	6	5	6	8	9	9	10	12	
Total	-	166	310	822	960	999	1200	1318	1097	1092	814	800	656	646	

NA = not available

Table 1.1 Usage and examples of pesticides according to its sub-category (according to Bohmont, 1990).

Pesticides	Functions	Chemical Example
Herbicides	Kill and control weeds in farms and golf courses, and for domestic use.	2,4-D , methiuron, butafenacil, monolinuron
Insecticides	Kill and control disease carrier insects and insects in farms and household usage, for example to control termites.	tricalcium arsenate, benfuracarb, pyrethrin II, alanycarb, butoxycarboxim
Rodenticides	Kill and control rodents in farms and for domestic use.	scilliroside, brodifacoum, potassium arsenite
Fungicides	Kill and control fungi that cause disease in crops.	butylamine, penthiopyrad, fluopicolide
Nematocides	Kill and control nematodes.	abamectin, benomyl, carbofuran, aldoxycarb

environmental problem, since it frequently leads to soil contamination and subsequent pollution of surface and ground water (Kobližek *et al.* 2002).

Currently, the Pesticides Act 1974 is the principal legislation for the control of pesticides in Malaysia. However, there is no guideline and regulation on how to use and handle (including disposal) pesticides and pesticides waste. A farmer can spray as much as he wishes as long as it is not a banned product. Pesticide waste can consist of the pesticide itself (such as old stocks, leftovers or spillage), packaging, diluted product, contaminated clothing or other materials and rinsing water. At present, the Environment Quality Act (Amendment) 1985 controls only pesticide wastes and effluents from factories. These scenarios have increased the concern over herbicides pollutions in our environments, especially in drinking water. In the European Union, a guideline has been introduced to control the content of toxic compound in the water source (Merz *et al.* 1996), which are  $0.1\mu\text{g/l}$  for one component and  $0.5\mu\text{g/l}$  for total amount of the active ingredients. Water sources exceeding the maximum concentrations of pollutant were considered very toxic and hazardous to life. Similar guideline should also be introduced to the Environment Quality Act.

However, the guideline alone is ineffective if no monitoring program is conducted. If herbicides contamination is not regularly monitored, public health will be at risk since several herbicides such as atrazine are carcinogenic to human and animals. In addition, some mild toxic pesticides are converted to other compounds which are toxic to target organism (Barrett, 1996). The monitoring program must be carried out continuously, especially the water reservoirs. Thus, there is a need for a new detection method that is suitable for such monitoring program to provide early-warning systems for effective monitor and control of the environment to minimize the risk associated with pesticides used (Hamada and Wintersteiger, 2002). Many methodologies have been adapted in research and field area to monitor and control

pesticide in the environment. A practical monitoring method requires rapid, simple, and low cost screening and detection procedures for detection of herbicides residue.

Current methodologies for pesticide detection, especially herbicides are not suitable for large-scale monitoring program. Most of the techniques (for example, HPLC and GC-MS) require large and expensive equipment and materials. In addition, these methods were tedious and complicated (Giardi *et al.* 2001). Only highly trained personal would be able to conduct the tests, resulting in cost increase.

In this research, we have developed a method for herbicide detection that can be used in a continuous monitoring system or as a large-scale monitoring program to monitor the level of herbicide in water samples as well as other aqueous samples. In order to monitor the herbicide contamination regularly, the method being developed has to be fast, easy to be carried out, and does not involve expensive materials or equipments because we will need to regularly analyze samples collected from the areas monitored. A method or technique that involves expensive instruments and solvents is not suitable for continuous monitoring because the cost of running just one single test would be high and time consuming, even though the technique could be highly sensitive. An inexpensive operating cost will allowed us to screen and analyze many samples collected from many field areas. Moreover, an “easy-to-handle” technique does not involve tedious procedure, thus the number of samples that can be analyzed in a period can be increased. Larger areas can be monitored instead of a few limited areas, thus lowering the risk of herbicides contaminant poisoning of human and other organisms.

Fluorometry was used as a detection tool in this research. Fluorometric detection is less expensive than HPLC or GC-MS. The detection was based on the emission of the chlorophyll fluorescence by *in vivo* microalgae chlorophyll pigments. In plant leaves, it has been shown that the fluorescence induction kinetics under constant illumination of appropriate intensity, provides a simple and inexpensive tool for monitoring the translocation and detoxification of PS II herbicides *in vivo* (Ducruet

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