Development & Evaluation of Chlorophyll *a* Fluorescence as a bioanalytical tool for pollutant identification

A thesis submitted for the degree of Doctor of Philosophy by

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This thesis is dedicated to my grandfather,
Geoff Murphy (1928-2009).

As a child, my grandfather's love and respect for the land, the bush and the ocean was a true inspiration for me to one day be able to do all that I can to help save our beautiful Earth.
Grandad – I hope you're proud that I've finished university and can finally get to work.

CERTIFICATE OF AUTHORSHIP/ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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Rachael Smith (PhD Candidate)

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ABSTRACT

There is potential to improve water quality monitoring programs by generating pollution data that better represents the aquatic ecosystem being monitored. By incorporating rapid and cost-effective bioanalytical methods into water quality monitoring programs, risk associated with unrepresentative data can be reduced by increasing the number of samples collected without incurring additional costs. The rapid and cost-effective toxin-identification method presented here is based on quantifying patterns of change in chlorophyll *a* fluorescence (fluorescence fingerprints) associated with a toxicants mode of action (MoA). Chlorophyll *a* fluorescence yield is influenced by environmental factors and can be used to identify stress caused by light, nutrient status and the presence of pollutants. When the functional state of the photosynthetic apparatus changes, the yield of fluorescence emission also changes, generating a chlorophyll *a* fluorescence response that has previously been thought to be unique based on a toxicants mode of action.

The toxin-identification method was developed as a bioanalytical system based on the chlorophyll a fluorescence responses of a microalgae (*Dunaliella tertiolecta*) to herbicide and nutrient impacts, measured using the Imaging-PAM fluorometer. The analysis of the fluorescence response was the novel method; a holistic approach was employed. Unlike previous approaches which measured one fluorescence parameter for toxicant identification, the method presented here assessed the temporal unity of change in energy dissipation, which was found to be unique depending on a chemical's mode of action (i.e. its physico-chemical properties and toxicokinetic relationship with the organism). The method was tested for two different uses: (1) as a non-specific biosensor able to identify herbicides (and their potency) in a water sample of unknown constituents, and (2) a method specific to the identification and potency of nutrients in a water sample.

Seven herbicides were examined totaling three different MoAs; PSII inhibitors (DCMU, Irgarol, Bromacil and Simazine), uncoupling of phosphorylation (Dinoseb and PCP) and creation of reactive oxygen species (paraquat). By first generating a database of reference response patterns, the response patterns of laboratory derived test samples were then measured and quantitatively compared to the reference

patterns. The unknown or test sample was compared to reference toxicants using a mean-square fit (MSF) software program. The MSF program tells the user how well the fingerprint of the test sample fits to each of the fingerprints of the reference chemicals. The method showed 93% accuracy in correctly identifying six herbicides, with false negative identifications occurring for only two toxicants, simazine (8% of samples) and Dinoseb (27% of samples).

Phosphate induced fluorescence transients were also assessed to demonstrate that the toxin-identification method was versatile in its ability to also be used as a selective biomarker. By culturing P-limited *D. tertiolecta* cells, a unique fluorescence response was recorded upon additions of PO_4^{3-}. The NIFT (nutrient induced fluorescent transient) response was specific to PO_4^{3-} additions compared to NH_4^{3+} and NO^{2-} additions. Quantification of the NIFT response showed high levels of precision and specificity for multiple fluorescence parameters.

The toxin-identification method presented here is still in its preliminary stages and higher levels of validation are still necessary including testing environmental samples, and comparing results from the toxin-identification method to results from chemical analysis. However, this thesis presents the foundational work of a unique and powerful bioanalytical tool with the potential to greatly improve water quality management practices.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ΔF_{max}	Maximum change in fluorescence
α_{max}	Slope to the maximum change in fluorescence
AICS	Australian Inventory of Chemical Substances
AL	Actinic Light
ALi	Actinic Light intensity
ALw	Actinic Light width
ANOVA	Analysis of Variance
ANZECC	Australian and New Zealand Environment and Conservation Council
APHA	American Public Health Association
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
AUC	Area under the whole curve
AUC_{max}	Integrated area under the curve from time 0 to time at ΔF_{max}
BP	Black Plate
CA	Concentration Addition
CCD	Charge-Coupled Device
CITB	Chemical Information and Testing Branch
CoV	Coefficient of Variation
CSIRO	Commonwealth Scientific and Industrial Research Organisation
D1	D1 protein
EC	Effective Concentrations
EQY	Effective Quantum Yield of PSII
EU	European Union
Fm	Maximum Fluorescence (dark)
Fm'	Maximum Fluorescence under photosynthetic active radiation
Fo	Minimum Fluorescence (dark)
Fo'	Minimal fluorescence yield of an illuminated sample, lowered with respect to Fo by non-photochemical quenching
Ft	Fluorescence yield determined under photosynthetic active radiation

Fv/Fm	Maximum quantum yield
GCMS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
I_k	Minimum actinic light level at which the maximum rate of rETR (rETRmax) occurs
ISO	International Standardization Organization
LCMS	Liquid Chromatography Mass Spectrometry
LED	Light-Emitting Diode
LoD	Limit of Detection
Log K_{ow}	Octanol/water partitioning coefficient
LoQ	Limit of Quantitation
ML	Measuring Light
MLf	Measuring Light frequency
MLi	Measuring Light intensity
MoA	Mode of Action
MSF	Mean-Square Fit
MWP	Microtiter Microfluor (Thermo Scientific) 96-well Plates
MWP+F	Microtiter Microfluor (Thermo Scientific) 96-well Plates with Filter
N-limited	Nitrogen limited
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NIFT	Nutrient Induced Fluorescent Transient
NPQ	Non-Photochemical Quenching
P-limited	Phosphorous limited
PAH	Polycyclic Aromatic Hydrocarbon
PAM	Pulse Amplitude Modulated
PAR	Photosynthetically Active Radiation
Pi	Inorganic phosphorous
pK_a	Ionisation constant
PSI	Photosystem I
PSII	Photosystem II
qL	Coefficient of photochemical quenching (based on 'lake' model)

qN	Coefficient of non-photochemical quenching
qP	Coefficient of photochemical quenching (based on 'puddle' model)
Q_A	Plastiquinone A
Q_B	Plastiquinone B
R^2	Coefficient of determination
rETR	Relative Electron Transport Rate
rETRmax	Maximum Rate of rETR
RLC	Rapid Light Curves
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
SD	Standard Deviation
SNR	Signal to Noise Ratio
SOP	Standard Operating Procedure
SP	Saturation Pulse
SPi	Saturation Pulse intensity
SPE	Solid Phase Extraction
TIE	Toxicity Identification Evaluation
USEPA	United States Environmental Protection Authority
WFD	Water Framework Directive
WP	White Plate
Y(NO)	Non-regulated non-photochemical quenching
Y(NPQ)	Regulated non-photochemical quenching