

# **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**

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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)

## ACKNOWLEDGEMENTS

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## ABSTRACT

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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  $\text{PO}_4^{3-}$ . The NIFT (nutrient induced fluorescent transient) response was specific to  $\text{PO}_4^{3-}$  additions compared to  $\text{NH}_4^{3+}$  and  $\text{NO}_2^-$  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

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CERTIFICATE OF AUTHORSHIP/ORIGINALITY	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT	vi
CONTENTS	viii
LIST OF FIGURES	xii
LIST OF TABLES	xv
LIST OF ABBREVIATIONS	xvii
<b>1.0 INTRODUCTION</b>	<b>2</b>
1.1 Pollution Monitoring	2
1.2 Pollutant Identification	6
1.3 Chlorophyll <i>a</i> Fluorescence Toxicology	9
1.3.1. Status of chlorophyll <i>a</i> fluorescence in environmental toxicology	9
1.3.2. Photosynthesis and photoinhibition	11
1.3.3. Measurement of toxicant induced photoinhibition with chlorophyll <i>a</i> fluorescence	19
1.3.4. Fluorescence parameters and a toxicants mode of action	22
1.4 Aim of Thesis: Development of a chlorophyll <i>a</i> fluorescence fingerprint for toxicant identification and potency estimation	23
1.5 Thesis Outline	26
1.6 References	28
<b>2.0 GENERAL METHODS</b>	<b>34</b>
2.1 Introduction	34
2.2 Bioanalytical System	34
2.2.1. Test organism	34
2.2.2. Imaging-PAM fluorometer	36
2.2.2.1. Components and set-up of the Maxi Imaging-PAM fluorometer	37
2.2.2.2. System operation	38
2.2.3. Chlorophyll <i>a</i> fluorescence analysis	39
2.3 Operating Procedure	42
2.3.1. Batch culturing and test preparation	42
2.3.2. Culture calibration and standardization	42
2.3.3. Cell density determination	43
2.3.4. Test solutions	43
2.3.5. Solvent controls	43
2.3.6. Fluorescence measurements	45
2.4 Mathematical and Statistical Analysis	46
2.4.1. Computer software	46
2.4.2. Standard dose-response curves and effective concentrations	47



2.4.3.	Coefficient of variance and Variance	47
2.4.4.	Parallel curves and potency estimation	48
2.4.5.	Method for determining parallelism of dose-response curves and potency estimation using the MSF software program	48
<b>2.5</b>	<b>References</b>	<b>54</b>

### **3.0 OPTIMISATION OF CHLOROPHYLL *a* FLUORESCENCE TOXICITY TESTING FOR TOXICANT IDENTIFICATION USING THE IMAGING-PAM FLUOROMETER** **58**

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<b>3.1</b>	<b>Introduction</b>	<b>58</b>
3.1.1.	Imaging-PAM fluorometer	58
3.1.2.	Multi-well plates	60
3.1.3.	Chapter aims	61
<b>3.2</b>	<b>Materials and Methods</b>	<b>63</b>
3.2.1.	Test chemicals	64
3.2.2.	Biomaterial	64
3.2.3.	Chlorophyll <i>a</i> fluorescence measurements	64
3.2.4.	Comparison of the light field within the wells of different types of 96-well plates	64
3.2.5.	Rapid light curves	65
3.2.6.	Test for optimal cell density	67
3.2.7.	Mathematical and statistical analysis	67
<b>3.3</b>	<b>Results</b>	<b>69</b>
3.3.1.	Stage 1: Heterogeneity of light field across 96-well plates	69
3.3.2.	Stage 2: Effect of well plate type on reflectance/attenuation of light	71
3.3.3.	Stage 3: Optimum cell density and Imaging-PAM setting selection for precision and sensitivity	75
<b>3.4</b>	<b>Discussion</b>	<b>77</b>
<b>3.5</b>	<b>References</b>	<b>81</b>
<b>3.6</b>	<b>Appendix</b>	<b>83</b>

### **4.0 TOXIN-IDENTIFICATION WITH CHLOROPHYLL *a* FLUORESCENCE** **85**

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<b>4.1</b>	<b>Introduction</b>	<b>85</b>
4.1.1.	Reaction rates	87
4.1.2.	Mode of action and fluorescence parameters	88
4.1.3.	Parallelism of dose-response curves	91
4.1.4.	Chapter aims	92
<b>4.2</b>	<b>Materials and Methods</b>	<b>94</b>
4.2.1.	Test chemicals	94
4.2.2.	Biomaterial	94
4.2.3.	Cell density	94
4.2.4.	Chlorophyll <i>a</i> fluorescence measurements and analysis	94
4.2.5.	Exposure experiments	94
4.2.6.	Mathematical and statistical analysis	95
4.2.7.	Calculation of reference input data and acceptance range	96
4.2.8.	Calculation of parallelism	96
<b>4.3</b>	<b>Results</b>	<b>100</b>

4.3.1.	Reference curves	100
4.3.2.	Test sample curves	101
4.3.3.	Acceptance ranges	103
4.3.4.	Toxicant prediction	103
4.3.5.	Toxicant identification using multiple fluorescence parameters	106
4.3.6.	Time-dependent variations	108
<b>4.4</b>	<b>Discussion</b>	<b>110</b>
<b>4.5</b>	<b>References</b>	<b>118</b>
<b>5.0</b>	<b>SINGLE POLLUTANT IDENTIFICATION FROM A MIXTURE</b>	<b>123</b>
<b>5.1</b>	<b>Introduction</b>	<b>123</b>
5.1.1.	Mixture toxicology	123
5.1.2.	Effects of mixtures on chlorophyll <i>a</i> fluorescence	125
5.1.3.	Chapter aims	127
<b>5.2</b>	<b>Materials and Methods</b>	<b>130</b>
5.2.1.	Test chemicals	130
5.2.2.	Biomaterial	130
5.2.3.	Cell density	130
5.2.4.	Chlorophyll <i>a</i> fluorescence measurements and analysis	130
5.2.5.	Exposure experiments	130
5.2.6.	Mathematical and statistical analysis	132
<b>5.3</b>	<b>Results</b>	<b>134</b>
5.3.1.	Chemical interactions of binary mixtures	134
5.3.2.	Parallelism of binary mixtures to single toxicant data	137
<b>5.4</b>	<b>Discussion</b>	<b>154</b>
<b>5.5</b>	<b>References</b>	<b>160</b>
<b>6.0</b>	<b>NUTRIENT INDUCED FLUORESCENCE TRANSIENTS (NIFTS): a SELECTIVE BIOMARKER FOR PO<sub>4</sub><sup>3-</sup> ASSESSMENT</b>	<b>163</b>
<b>6.1</b>	<b>Introduction</b>	<b>163</b>
6.1.1.	Physiological changes associated with nutrient limitation and Nutrient Induced Fluorescence Transients (NIFTs)	164
6.1.2.	Chapter aims	166
<b>6.2</b>	<b>Materials and Methods</b>	<b>167</b>
6.2.1.	Test chemicals	167
6.2.2.	Biomaterial	167
6.2.3.	Phosphate-limited and P-replete cultures	167
6.2.4.	Chlorophyll <i>a</i> fluorescence measurements and analysis	168
6.2.5.	Exposure experiments	168
6.2.6.	Mathematical and statistical analysis	169
<b>6.3</b>	<b>Results</b>	<b>172</b>
6.3.1.	Chlorophyll <i>a</i> fluorescence changes associated with PO <sub>4</sub> <sup>3-</sup> limitation	172
6.3.2.	End-point validation	175
6.3.3.	Selectivity	178
6.3.4.	Effect of the presence of another pollutant on PO <sub>4</sub> <sup>3-</sup> relative potency estimations	179
<b>6.4</b>	<b>Discussion</b>	<b>184</b>

6.5	References	189
<b>7.0</b>	<b>DISCUSSION</b>	<b>193</b>
7.1	Purpose of the Method	193
7.2	The Developed Method	194
7.3	Applying the Method: Types of Chemicals and Matrices	197
7.4	Standard Operating Procedure	200
7.5	Analytical Requirement: Evaluation of Method Performance Characteristics	204
7.6	Concluding Remarks	205
7.7	References	206

## LIST OF FIGURES

Figure 1.1: Fluorescence induction or 'Kautsky' curve based on the PAM technique. A time course of changes in fluorescence upon illumination of ML (measuring light), SP (saturation pulse) and AL (actinic light) records the fluorescence parameters Fo (minimum fluorescence), Fm (maximum fluorescence), Ft (minimum fluorescence under photosynthetic active radiation) and Fm' (maximum fluorescence under photosynthetic active radiation).....	20
Figure 1.2: Hierarchy of stages of the objectives and requirements for prevalidation, validation and standardization of new analytical methods. RSD=relative standard deviation; CV=coefficient of variation. Adapted from Taverniers, De Loose and Van Bockstaele (2004).....	25
Figure 2.1: The Maxi-Imaging-PAM; (a) a schematic representation of the Maxi-Imaging-PAM (adapted from Walz 2009), (b) photographic picture showing the configuration of the LED lamps and CCD camera objective lens (Source: Walz 2009), and (c) a 2D image of chlorophyll fluorescence measured from a 96-well plate.....	38
Figure 2.2: Changes in fluorescence of <i>Dunaliella tertiolecta</i> when spiked with controls (Milli-Q water) compared to solvent controls (NaOH + methanol). The x-axis indicates fluorescence changes of three fluorescence parameters; EQY, Y(NPQ) and Y(NO), at two exposure periods; 0.5 h and 2 h. Bars indicate average values $\pm$ standard error of mean (n = 66 and 22 for controls and solvent controls, respectively). * indicates where a significant difference ( $p \leq 0.05$ ) lies between Milli-Q and solvent controls.....	45
Figure 2.3: Parallel shifts of a concentration-effect curve to the right along the x-axis (blue curve) and up along the y-axis (red curve).....	50
Figure 2.4: Log dose-response curves of substances S1, S2 and S3 from Table 2.2.....	52
Figure 2.5: Example of input data required for the MSF software program and the subsequent output data calculated from the MSF software program. Highlighted areas demonstrate the mean-square fit (or estimation of parallelism) between the S1 and S2 dose-response curves and the potency estimation of S2 compared to S1.....	53
Figure 3.1: Flow diagram of experimental stages applied in this chapter in order to determine the settings and designations (i.e. plate type, plate testing region and cell density) to be used in the experimental methods for Chapters 4 – 6.....	63
Figure 3.2: Divided regions of a 96-well plate; Region 1- middle 4 wells, Region 2- middle 16 wells, Region 3- middle 36 wells, Region 4- middle 48 wells and Region 5- all 96-wells.....	69
Figure 3.3: Effect of <i>D. tertiolecta</i> cell density on EC50 concentrations based on the % change in EQY by DCMU ( $\mu$ M) tested with black 96-well plates (●) and Microtiter white plates + filter (○). Symbols and error bars represent mean $\pm$ 1 S.D. (n=3).....	76
Figure 3.4: Depiction of the light fields (reflection and absorption) within the MWP+F and BP wells measured by a spherical light sensor. Blue arrows indicate wavelengths of light emitted by the Imaging-PAM fluorometer.....	79
Figure 3.5: Depiction of the light fields and chlorophyll a fluorescence of microalgal cells in the MWP+F and BP. Blue region represents light intensity emitted by the Imaging-PAM fluorometer, green dots indicate microalgae not emitting fluorescence and red dots indicate microalgae emitting fluorescence.....	79
Figure 4.1: From left to right; chemical characteristics of toxicants impacting on the light reactions of the photosynthetic apparatus, their identifying characteristics (based on pharmacological (A), chlorophyll a fluorescence (B) and toxicological (C) methods, and the measurable responses that, when combined (D), will theoretically produce a unique fluorescence fingerprint.....	87
Figure 4.2: Methodology for calculation of the reference input data and acceptance range for toxicant reference samples. This process accounts for data of only one fluorescence parameter for an individual toxicant. The process is repeated for each toxicant and each fluorescence parameter.....	99
Figure 4.3: Methodology for calculation of parallelism of test samples with reference data of each toxicant. This process accounts for data of only one fluorescence parameter for an individual test sample. The process is repeated for each test sample and each fluorescence parameter.....	99
Figure 4.4: Box-plot of R <sup>2</sup> values of EQY, Y(NPQ) and Y(NO) at 30 minutes and 2 h dose-response curves for (a) DCMU, (b) Irgarol 1051, (c) Bromacil, (d) Simazine, (e) PCP, (f) Dinoseb, and (g) Paraquat. Top, middle and bottom lines of box indicate the 75 <sup>th</sup> percentile, median and 25 <sup>th</sup> percentile, error bars above and below the box indicate the 90 <sup>th</sup> and 10 <sup>th</sup> percentiles (n=12).....	102
Figure 4.5: Dose-response changes in EQY of <i>D. tertiolecta</i> exposed to the PSII inhibitors; DCMU, Simazine, Irgarol 1051 and Bromacil for 30 minutes (●) and 2 h (○). Values represent mean % change in EQY from control ( $\pm$ 1 S.D.), n=12.....	109

Figure 5.1: Isobologram (adapted from Altenburger et al. 1993) illustrating three types of joint action of a binary mixture: additive, antagonistic and synergistic. Isoboles are lines representing a defined effect level and indicate the mixture ratios of the two substances (S1 and S2) required to achieve that effect level. The straight line indicates additivity, an upward-bent line indicates antagonism and a downward-bent line indicates synergism. ....	129
Figure 5.2: Experimental design of a 6·6 dose-combination matrix for a binary mixture of substances 1 and 2 at various concentrations ( $C_0, \dots, C_6$ ) (adapted from Altenburger et al. 2003). Circles represent dose-combinations. Green horizontal lines represent an n·n experimental design where various concentrations of Substance 1 (S1) as the primary toxicant are tested with a fixed concentration of Substance 2 (S2) as the secondary toxicant: S1(1°) + S2 (2°). Yellow lines represent the converse, i.e. the dose-combinations when S2 is the primary toxicant with fixed concentrations of S1 as the secondary toxicant: S2(1°) + S1(2°). Dose-combinations for the ray experimental design are represented by red circles. Black lines represent single toxicant reference data for S1 (horizontal line) and S2 (vertical line). ....	132
Figure 5.3: Isobolic representations of binary mixture dose-response relationships illustrating (a) concentration addition, (b) antagonism, and (c) dose level-dependent deviation. Lines and values indicate effect concentrations, S1 and S2 are substances 1 and 2 of the binary mixture (adapted from Jonker et al. 2005). ....	133
Figure 5.4: Isobolic representation of the % mean change in (a) EQY, and (b) Y(NO) for the 6·6 matrix of DCMU + Bromacil dose combinations; values indicate response levels (n=4). ....	135
Figure 5.5: Isobolic representation of the % mean change in (a) EQY, and (b) Y(NPQ) for the 6·6 matrix of Dinoseb + PCP dose combinations; values indicate response levels (n=4). ....	135
Figure 5.6: Isobolic representation of the % mean change in (a) EQY, (b) Y(NO), and (c) Y(NPQ) using a 6·6 matrix of DCMU + PCP dose combinations; values indicate response levels (n=4). ....	137
Figure 5.7: Average mean-square fits (n = 4) of primary toxicant dose-response curves with increasing additions of the secondary toxicant fitted to both the primary and secondary toxicant reference data. Figures on the left represent DCMU (●) as the primary toxicant with additions of Bromacil (●) as secondary toxicant for the fluorescence parameters (a) EQY and (c) Y(NO). Figures on the right represent Bromacil (●) as primary toxicant and DCMU (●) as secondary toxicant for the fluorescence parameters (b) EQY and (d) Y(NO). Dotted lines represent acceptance range derived from single toxicant reference data for DCMU (black) and Bromacil (red). Solid lines represent regression lines for DCMU data (black) and Bromacil data (red). ....	139
Figure 5.8: Changes in EQY of DCMU + Bromacil mixtures based on the n·n experimental design: (a) DCMU (1°) + Bromacil (2°), 0.25 and 1.0 μM Bromacil, compared to DCMU (red line) and Bromacil (inset) single toxicant reference curves; and (b) Bromacil (1°) + DCMU (2°), 0.02 and 0.04 μM DCMU, compared to Bromacil (red line) and DCMU (inset) single toxicant reference curves. Values represent mean ± 1 S.D. (n = 4). ....	141
Figure 5.9: DCMU:Bromacil mixture dose-response curves (●) based on the ray design, and DCMU (▼) and Bromacil (■) single toxicant reference curves for the fluorescence parameters (a) EQY and (b) Y(NO). Values represent mean ± 1 S.D. (n = 4). ....	142
Figure 5.10: Average mean-square fits (n = 4) of primary toxicant dose-response curves with increasing additions of the secondary toxicant fitted to both the primary and secondary toxicant reference data. Figures on the left represent Dinoseb (●) as the primary toxicant with additions of PCP (●) as secondary toxicant for the fluorescence parameters (a) EQY and (c) Y(NO). Figures on the right represent PCP (●) as primary toxicant and Dinoseb (●) as secondary toxicant for the fluorescence parameters (b) EQY and (d) Y(NO). Dotted lines represent acceptance range derived from single toxicant reference data for Dinoseb (black) and PCP (red). Solid lines represent regression lines for Dinoseb data (black) and PCP data (red). ....	144
Figure 5.11: Changes in EQY of Dinoseb + PCP mixtures based on the n·n experimental design: (a) Dinoseb (1°) + PCP (2°), 10 and 25 μM PCP, compared to Dinoseb (red line) and PCP (inset) single toxicant reference curves; and (b) PCP (1°) + Dinoseb (2°), 0.02 and 0.04 μM Dinoseb, compared to PCP (red line) and Dinoseb (inset) single toxicant reference curves. Values represent mean ± 1 S.D. (n = 4). ....	146
Figure 5.12: Dinoseb:PCP mixture dose-response curves (●) based on the ray design, and Dinoseb (▼) and PCP (■) single toxicant reference curves for the fluorescence parameters (a) EQY and (b) Y(NPQ). Values represent mean ± 1 S.D. (n = 4). ....	147
Figure 5.13: Average mean-square fits (n = 4) of primary toxicant dose-response curves with increasing additions of the secondary toxicant fitted to both the primary and secondary toxicant reference data. Figures on the left represent DCMU (●) as the primary toxicant with additions of PCP (●) as secondary toxicant for the fluorescence parameters (a) EQY and (c) Y(NO). Figures on the right	

represent PCP (●) as primary toxicant and DCMU (●) as secondary toxicant for the fluorescence parameters (b) EQY and (d) Y(NPQ). Dotted lines represent acceptance range derived from single toxicant reference data for DCMU (black) and PCP (red). Solid lines represent regression lines for DCMU data (black) and PCP data (red). .....	149
Figure 5.14: Changes in Y(NO) and Y(NPQ) of DCMU + PCP mixtures based on the n.n experimental design: (a) change in Y(NO) of DCMU (1%) + PCP (2%), 10 and 50 μM PCP, compared to DCMU (red line) and PCP (inset) single toxicant reference curves; and, (b) change in Y(NPQ) of PCP (1%) + DCMU (2%), 0.02 and 0.08 μM DCMU, compared to PCP (red line) and DCMU (inset) single toxicant reference curves. Values represent mean ± 1 S.D. (n = 4).....	151
Figure 5.15: DCMU:PCP mixture dose-response curves (●) based on the ray design, and DCMU (▼) and PCP (■) single toxicant reference curves for the fluorescence parameters (a) EQY, (b) Y(NO) and (c) Y(NPQ). Values represent mean ± 1 S.D. (n = 4).....	153
Figure 6.1: A single NIFT response (Fm') of P-limited <i>D. tertiolecta</i> to PO <sub>4</sub> <sup>3-</sup> (8 μM). Lower case letters indicate points at which descriptive curve variables are calculated (see Table 6.1). .....	170
Figure 6.2: Fluorescence yields, EQY, Y(NO) and Y(NPQ), of <i>D. tertiolecta</i> cultured in P-replete and P-limited growth media, (n = 9, ± 1 S.D.). * indicates significant differences (p≤0.05) between P-replete and P-deplete fluorescence yields for individual fluorescence parameters. ....	173
Figure 6.3: Representative set of NIFT responses of P-limited <i>D. tertiolecta</i> to a range of PO <sub>4</sub> <sup>3-</sup> concentrations (0.5 – 8.0 μM) for the fluorescence parameters: Fm', Ft, EQY, Y(NO), Y(NPQ) and NPQ/4.....	174
Figure 6.4: Representative set of NIFT responses of P-limited <i>D. tertiolecta</i> to (a) DCMU (0.01 μM), (b) PO <sub>4</sub> <sup>3-</sup> (0.1 μM) and (c) PO <sub>4</sub> <sup>3-</sup> (0.1 μM) + DCMU (0.01 μM) for the fluorescence parameters: Ft, Fm', Y(NO) and NPQ/4.....	180
Figure 6.5: Representative set of NIFT responses of P-limited <i>D. tertiolecta</i> to (a) Cu <sup>2+</sup> (10 μM), (b) PO <sub>4</sub> <sup>3-</sup> (0.1 μM) and (c) PO <sub>4</sub> <sup>3-</sup> (0.1 μM) + Cu <sup>2+</sup> (10 μM) for the fluorescence parameters: Ft, Fm', Y(NO) and NPQ/4. ....	182
Figure 7.1: Cog graphs representing the levels of measurement of the test procedure that (a) were used in this thesis and (b) theoretically could be used to account for a greater range of toxicants. Cogs represent (from bottom to top): concentrations of the chemical/sample, fluorescence parameters, exposure period, light intensity and test species. ....	201
Figure 7.2: 3D fingerprints of data generated from experiments in Chapter 4. Both fingerprints show the % changes in EQY (colour code) over time for a series of seven dilutions. The fingerprint for DCMU (left) shows clear differences in the fingerprint for PCP (right). .....	203

## LIST OF TABLES

Table 1.1: List of chemicals known to impact on the photosynthetic apparatus, mode of action and chlorophyll a fluorescence response.....	16
Table 2.1: Test chemicals and concentrations of the solvents used in stock solutions.....	44
Table 2.2: Serial dilutions and effect of two substances, S1, S2 and S3.....	51
Table 3.1: Variance of actinic light intensity supplied by the Imaging-PAM fluorometer across wells of four types of 96-well plates ( $\pm 1$ S.D., $n=3$ ). Regions of wells are based on Figure 3.1.....	70
Table 3.2: Percent change in light intensity recorded within the middle four wells of four different 96-well plate types. Nineteen AL levels were tested and compared to the actual LED actinic light intensity of the Imaging-PAM. Values represent the mean % change ( $\pm 1$ S.D.) of light intensities within wells from the actual LED actinic light intensities ( $n = 4$ ). .....	71
Table 3.3: Minimum saturating irradiance ( $I_k$ ) values were calculated from rapid light curves of <i>D. tertiolecta</i> tested in four different types of 96-well plates. Values represent the mean ( $\pm 1$ S.D.) LED actinic light intensity of the Imaging-PAM fluorometer at which rETR is reached ( $n=3$ ). .....	72
Table 3.4: Cell densities of <i>Dunaliella tertiolecta</i> and Imaging-PAM settings for the black 96-well plate and Microtiter white plate + filter required to to obtain an Ft reading of $\sim 0.15$ . .....	75
Table 3.5: Coefficients of variation (%) of <i>D. tertiolecta</i> DCMU (1 - EC50) concentrations ( $\mu\text{M}$ ) at different cell densities tested in black 96-well plates and Microtiter white plates + filter.....	77
Table 3.6: Multi-well plate and Imaging-PAM specifications for use in the toxicant identification bioassay.....	80
Table 4.1: Physico-chemical properties of test chemicals and their mode of action (MoA). .....	98
Table 4.2: $R^2$ values of reference data to a 4-parameter sigmoid regression model ( $n=12$ ). .....	100
Table 4.3: Acceptance ranges of toxicant reference data for the fluorescence parameters EQY, Y(NPQ) and Y(NO). Values represent average mean-square fit ( $\pm 1$ S.D.), $n = 12$ , n/a indicate where reference data did not fit the 4-parameter sigmoid regression model (see Table 4.2). .....	103
Table 4.4: Identification of toxicant test samples using parallelism of dose response curves for the fluorescence parameter EQY after a 30 minute exposure. Values are expressed as % ( $n=12$ ), values in bold indicate a correct positive match, values either side of these are false positive errors. Acceptance range= Mean + 1 S.D. ....	104
Table 4.5: Identification of toxicant test samples using parallelism of dose response curves for the fluorescence parameter EQY after a 2 hour exposure. Values are expressed as % ( $n=12$ ), values in bold indicate a correct positive match, values either side of these are false positive errors. Acceptance range= Mean + 1 S.D. ....	105
Table 4.6: Identification of toxicant test samples using parallelism of dose response curves for the fluorescence parameter Y(NPQ) after a 30 minute and 2 hour exposure. Values are expressed as % ( $n=12$ ), values in bold indicate a correct positive match, values either side of these are false positive errors. Acceptance range= Mean + 1 S.D. ....	105
Table 4.7: Identification of toxicant test samples using parallelism of dose response curves for the fluorescence parameter Y(NO) after a 30 minute exposure. Values are expressed as % ( $n=12$ ), values in bold indicate a correct positive match, values either side of these are false positive errors. Acceptance range= Mean + 1 S.D. ....	105
Table 4.8: Identification of toxicant test samples using parallelism of dose response curves for the fluorescence parameter Y(NO) after a 2 hour exposure. Values are expressed as % ( $n=12$ ), values in bold indicate a correct positive match, values either side of these are false positive errors. Acceptance range= Mean + 1 S.D. ....	106
Table 4.9: Identification of toxicant test samples using composite response analysis based on the parallelism of dose response curves with an acceptance range of Mean + 1 S.D. for the fluorescence parameters, EQY, Y(NPQ) and Y(NO) after a 30 minutes and 2 hour exposure. Values are expressed as % ( $n=12$ ), values in bold indicate a correct positive match, values either side of these are false positive errors.....	107
Table 4.10: Identification of toxicant test samples using a composite response analysis based on the parallelism of dose response curves with an acceptance range of Mean + 2 S.D. for the fluorescence parameters, EQY, Y(NPQ) and Y(NO) after a 30 minutes and 2 hour exposure. Values are expressed as % ( $n=12$ ), values in bold indicate a correct positive match, values either side of these are false positive errors. ....	107
Table 4.11: Percent of false negative predictions for the fluorescence parameter EQY at 30 minutes and 2 h exposures and the composite response analysis with acceptance ranges of mean + 1 S.D. and mean + 2 S.D., $n = 12$ . .....	108

Table 4.12: Mean EC30 values ( $\pm 1$ S.D.) calculated from DCMU, Simazine, Irgarol 1051 and Bromacil dose-response curves after 30 minute and 2 h exposures to <i>D. tertiolecta</i> .	109
Table 5.1: Mean-square values for DCMU:Bromacil mixture dilution curve fitted to DCMU and Bromacil reference curves for the fluorescence parameters EQY and Y(NO), and test for parallelism.	143
Table 5.2: Average ( $\pm 1$ S.D., $n=4$ ) mean-square values for Dinoseb:PCP mixture dose-response curve fitted to Dinoseb and PCP single toxicant reference curves for the fluorescence parameters EQY and Y(NPQ), and test for parallelism.	148
Table 5.3: Mean-square values for DCMU:PCP mixture dilution curve fitted to DCMU and PCP reference curves for the fluorescence parameters EQY, Y(NPQ) and Y(NO), and test for parallelism.	153
Table 6.1: Descriptive curve variables of a NIFT response of P-limited <i>D. tertiolecta</i> to $PO_4^{3-}$ , calculated from points on the curve (a, b, c, d) depicted in Figure 6.1.	170
Table 6.2: Average $PO_4^{3-}$ potency estimates of $PO_4^{3-}$ reference samples fitted to a $PO_4^{3-}$ reference standard ( $n = 3$ ).	176
Table 6.3: Coefficient of Variation of $PO_4^{3-}$ potency estimates presented in Table 6.2	176
Table 6.4: Acceptance statistic (%) of $PO_4^{3-}$ test dose-response data with a parallel fit to the $PO_4^{3-}$ reference standard.	177
Table 6.5: Average $PO_4^{3-}$ potency estimates of $PO_4^{3-}$ test samples fitted to a $PO_4^{3-}$ reference standard ( $n=6$ ).	177
Table 6.6: Coefficient of Variation of $PO_4^{3-}$ potency estimates presented in Table 6.5.	178
Table 6.7: Significant differences ( $p \geq 0.05$ ) of the maximum change in Ft and Fm' for P-limited <i>D. tertiolecta</i> cultures with additions of $PO_4^{3-}$ , $NH_4^+$ and $NO_3^{2-}$ , and for P-replete <i>D. tertiolecta</i> cultures with $PO_4^{3-}$ additions compared to Milli-Q control data.	178
Table 6.8: Average relative potency estimation and acceptance statistic of $PO_4^{3-}$ + DCMU mixtures dose-response data fitted to a $PO_4^{3-}$ reference standard ( $n=3$ ). P-values of paired samples t-test ( $df = 1$ ) of potency estimates of $PO_4^{3-}$ /DCMU mixtures compared to $PO_4^{3-}$ controls.	181
Table 6.9: Average relative potency estimation and parallel acceptance statistic of $PO_4^{3-}$ /Cu <sup>2+</sup> mixtures dose-response data fitted to a $PO_4^{3-}$ reference standard ( $n=3$ ). P-values of paired samples t-test ( $df = 1$ ) of potency estimates of $PO_4^{3-}$ /Cu <sup>2+</sup> mixtures compared to $PO_4^{3-}$ controls.	183



## LIST OF ABBREVIATIONS

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$\Delta F_{\max}$	Maximum change in fluorescence
$\alpha_{\max}$	Slope to the maximum change in fluorescence
<b>AICS</b>	Australian Inventory of Chemical Substances
<b>AL</b>	Actinic Light
<b>ALi</b>	Actinic Light intensity
<b>ALw</b>	Actinic Light width
<b>ANOVA</b>	Analysis of Variance
<b>ANZECC</b>	Australian and New Zealand Environment and Conservation Council
<b>APHA</b>	American Public Health Association
<b>ATP</b>	Adenosine Triphosphate
<b>ATPase</b>	Adenosine Triphosphatase
<b>AUC</b>	Area under the whole curve
$AUC_{\max}$	Integrated area under the curve from time 0 to time at $\Delta F_{\max}$
<b>BP</b>	Black Plate
<b>CA</b>	Concentration Addition
<b>CCD</b>	Charge-Coupled Device
<b>CITB</b>	Chemical Information and Testing Branch
<b>CoV</b>	Coefficient of Variation
<b>CSIRO</b>	Commonwealth Scientific and Industrial Research Organisation
<b>D1</b>	D1 protein
<b>EC</b>	Effective Concentrations
<b>EQY</b>	Effective Quantum Yield of PSII
<b>EU</b>	European Union
<b>F<sub>m</sub></b>	Maximum Fluorescence (dark)
<b>F<sub>m</sub>'</b>	Maximum Fluorescence under photosynthetic active radiation
<b>F<sub>o</sub></b>	Minimum Fluorescence (dark)
<b>F<sub>o</sub>'</b>	Minimal fluorescence yield of an illuminated sample, lowered with respect to F <sub>o</sub> by non-photochemical quenching
<b>F<sub>t</sub></b>	Fluorescence yield determined under photosynthetic active radiation

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

<b>q<sub>N</sub></b>	Coefficient of non-photochemical quenching
<b>q<sub>P</sub></b>	Coefficient of photochemical quenching (based on ‘puddle’ model)
<b>Q<sub>A</sub></b>	Plastiquinone A
<b>Q<sub>B</sub></b>	Plastiquinone B
<b>R<sup>2</sup></b>	Coefficient of determination
<b>rETR</b>	Relative Electron Transport Rate
<b>rETR<sub>max</sub></b>	Maximum Rate of rETR
<b>RLC</b>	Rapid Light Curves
<b>ROS</b>	Reactive Oxygen Species
<b>RSD</b>	Relative Standard Deviation
<b>SD</b>	Standard Deviation
<b>SNR</b>	Signal to Noise Ratio
<b>SOP</b>	Standard Operating Procedure
<b>SP</b>	Saturation Pulse
<b>SP<sub>i</sub></b>	Saturation Pulse intensity
<b>SPE</b>	Solid Phase Extraction
<b>TIE</b>	Toxicity Identification Evaluation
<b>USEPA</b>	United States Environmental Protection Authority
<b>WFD</b>	Water Framework Directive
<b>WP</b>	White Plate
<b>Y(NO)</b>	Non-regulated non-photochemical quenching
<b>Y(NPQ)</b>	Regulated non-photochemical quenching